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Investigating the molecular mechanisms of pathogenic Group B *Streptococcus* interactions with fungus *Candida albicans*

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Abstract

Group B *Streptococcus* (GBS) is the leading cause of neonatal sepsis and meningitis in developed countries. In the majority of cases, GBS is vertically transmitted from the mother during or preceding birth. *Candida albicans* is an opportunistic fungal pathogen of the female GU tract, causing vaginal thrush, for which pregnancy is a risk factor. As *C. albicans* is known to synergistically interact with *Streptococcus* bacteria within the oral cavity, it was hypothesised that *C. albicans* and GBS may interact within the vaginal tract. Using a vaginal epithelial cell association assay, it was shown that *C. albicans* significantly promoted GBS association with vaginal epithelial cells (VECs) and, likewise, GBS significantly promoted *C. albicans*. The AgI/II family surface-expressed adhesins of GBS, designated Bsp proteins, were found to contribute to GBS interactions with VECs and with *C. albicans*, while the *C. albicans* cell surface protein Als3 was pivotal for the coassociation between these species. Investigations into the VEC response to GBS and *C. albicans* implied that coassociation may reduce neutrophil chemotaxis, despite enhanced transcription of proinflammatory cytokine genes. Proteomics studies revealed that extracellular matrix (ECM) components, or proteins that modulate ECM components, were significantly elevated in dual-species-infected VECs, while apoptosis-related proteins and proteins involved in MAPK signalling were largely downregulated. Taken together, these data suggest that GBS and *C. albicans* synergistically interact in a manner that could promote GU tract colonisation and persistence, and that this coassociation is dependent on *C. albicans* Als3 and partially dependent on GBS Bsp proteins.

Dedication

I dedicate this thesis to my family, and to Chris.

To my family, thank you for your unwavering support and advice. To mum and dad, thank you for believing in me and motivating me. To Amy, thank you for your excellent advice and encouragement.

Last, but by no means least, thank you to Chris for keeping me going in what has been a very difficult year.

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Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: DATE:

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Abbreviations

AgI/II	Antigen I/II
Als	Agglutinin-like sequence
AspA	Group A *Streptococcus* AgI/II protein
ATCC	American Type Culture Collection
BBB	Blood brain barrier
BSA	Bovine serum albumin
Bsp	Group B *Streptococcus* AgI/II protein
BV	Bacterial vaginosis
Ca	*Candida albicans*
CC	Clonal complex
CDC	Centre for Disease Control
cDNA	Complementary DNA
CFU	Colony forming unit
CFU/mL	Colony forming unit per mL
CNS	Central nervous system
CPS	Capsular polysaccharide
CVF	Cervicovaginal fluid
dH_2O	Distilled H_2O
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleotides
ECC	Early childhood caries
ECM	Extracellular matrix

EMT	Epithelial-mesenchymal transition
EOD	Early onset disease
EPS	Extracellular polymeric substance
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FMI	Forward migration index
FMLP	N-Formylmethionine-leucyl-phenylalanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
GI	Gastrointestinal
GM17	M17 media supplemented with glucose
Gp-340	Glycoprotein-340
GU	Genitourinary
HBMEC	Human brain microvascular endothelial cell
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
Hwp1	Hyphal wall protein 1
IAP	Intravenous antimicrobial prophylaxis
ICU	Intensive care unit
K-SFM	Keratinocyte serum-free medium
LB	Luria-Bertani media
LDH	Lactate dehydrogenase
LOD	Late onset disease
LTA	Lipoteichoic acid
MKP1	Mitogen-activated protein kinase phosphatase 1
MOI	Multiplicity of infection

NK cells	Natural killer cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Pilus island
PIC	Protease inhibitor cocktail
PMSF	Phenylmethane sulonyl fluoride
RD2	Region of difference 2
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse-transcriptase PCR
SAB	Sabouraud dextrose
SAP	Secreted aspartyl protease
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
S-IgA	Secretory IgA
SpaP	*S. mutans* AgI/II protein
SRR	Serine-rich repeat protein
Srr-1	Serine-rich repeat protein-1
SspA/B	*S. gordonii* AgI/II proteins
ST	Sequence type
TBS	Tris-buffered saline
TBST	Tris-buffered saline with 0.1% Tween-20

THY	Broth for culturing GBS (Todd Hewitt powder with yeast extract)
TLR	Toll-like receptor
TRITC	Tetramethylrhodamine
VEC	Vaginal epithelial cell
VVC	Vulvovaginal candidiasis
WT	Wild type
YNBPT	Broth made up of yeast nitrogen base, tryptone, and Na_2HPO_4 adjusted to pH 7 with KH_2PO_4
YNBPTG	YNBPT with glucose
YPD	Broth for culturing *C. albicans* (Yeast extract, mycological peptone and glucose)
β-H/C	Beta-haemolysin/cytolysin

Chapter 1 Introduction

1.1 Vaginal epithelium overview

The vagina is made up of non-keratinised squamous epithelium and can be split into a number of sections. Above the basement membrane is the basal layer, followed by the parabasal layer, then the intermediate cells (stratum spinosum), with the superficial layer exposed at the surface (Figure 1-1). The superficial layer of cells is continuously exfoliated and replaced by cells from the basal layer of the epithelium. Basal cells flatten and differentiate as they travel through the layers of the vaginal epithelium to the superficial layer, with this process directed by hormones. As levels of oestrogen increase throughout the menstrual cycle, the volume of vaginal secretions increases, alongside the maturation and thickness of the epithelial layer, stimulating accretion of glycogen (Nunn and Forney, 2016;Sjoberg et al., 1988).

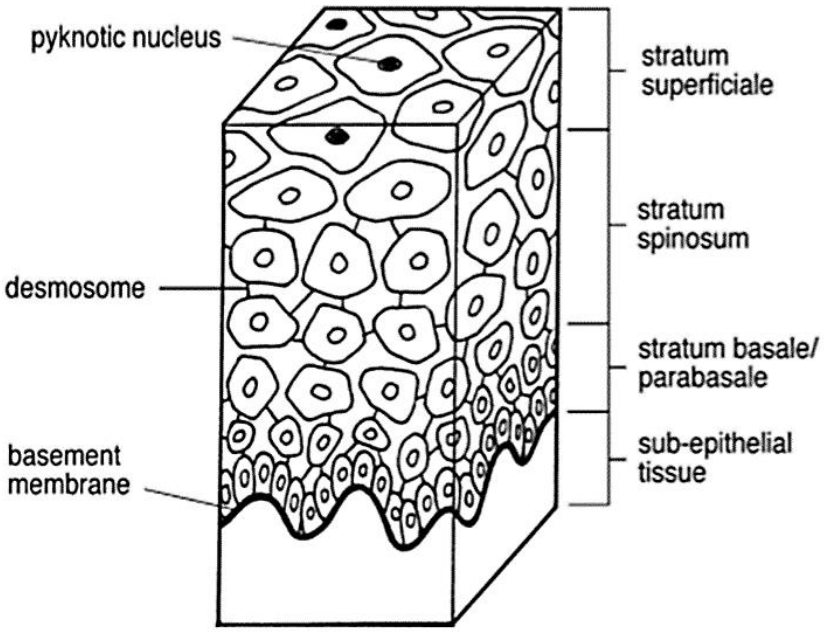

Figure 1-1 Vaginal epithelium structure.

Reproduced from (Farage and Maibach, 2006).

The female genitourinary (GU) tract undergoes changes throughout a woman's lifetime due to a number of factors, including hormones. For example, changes occur during the monthly menstrual cycle, at the onset of puberty or menopause (Farage and Maibach, 2006). At the start of the menstrual cycle, vaginal pH is typically higher than when in the

middle of the menstrual cycle (pH 6.6 on day 2 vs. pH 4.2 on day 14) (Wagner and Ottesen, 1982). Mid-cycle is also when the vaginal mucosa is at its thickest, with the highest level of intracellular glycogen (Farage and Maibach, 2006). Changes in the vaginal mucosa during menstruation are due to changes in hormone cycling, and this response is due to the oestrogen level gradually increasing up to mid-cycle. Decreasing oestrogen from this point influences exfoliation of the surface cells and thinning of the epithelium (Farage and Maibach, 2006). Levels of oestrogen are low (<50 pg/mL) during menstruation, achieving a first peak during ovulation (200-250 pg/mL) before decreasing and then building to a second, lesser peak around day 21 (150 pg/mL) (Gajer et al., 2012). These changes in oestrogen levels correlate with glycogen content within the vaginal tissues (Sjoberg et al., 1988).

The vaginal mucosal layer also provides receptors for microbial attachment. Vaginal epithelial cell (VEC) surface-expressed receptors facilitate microbial adhesion. For example, mannose receptor has been shown to enable binding of HIV protein gp120 (Fanibunda et al., 2011). Extracellular matrix (ECM) components, such as collagen, fibronectin and laminin are further adhesion targets for GU tract microorganisms, including *Trichomonas vaginalis*, *Chlamydia trachomatis*, *Lactobacillus crispatus*, *Lactobacillus brevis*, *Candida albicans* and Group B *Streptococcus* (GBS) (Singh et al., 2012;Skerl et al., 1984). Pre-incubation of *C. albicans* with fibronectin significantly reduced *C. albicans* binding of VECs, intimating a role for fibronectin in adhesion of *C. albicans* (Skerl et al., 1984). Additionally, one study found that all clinical isolates of GBS expressed laminin-binding protein Lmb and fibronectin-binding protein ScpB, indicating that binding these ECM components may be important for initiation of colonisation (Al Safadi et al., 2010). *Lactobacillus iners* binds to vaginal fibronectin more strongly than other species of lactobacilli, and this was suggested to contribute towards persistence of the microorganism (McMillan et al., 2013). Vaginal pH plays a role in adhesion of microbes, as many lactobacilli lose the ability to adhere to fibronectin as pH approaches neutral (Nagy et al., 1992). This may explain dysbiosis resulting from vaginal infections such as bacterial vaginosis (BV), during which the vaginal pH rises. Adhesion of *Gardnerella vaginalis*, a causative organism ofBV, is also pH dependent, with optimal pH being between 5 and 6 (Peeters and Piot, 1985).

1.2 Genitourinary tract in health and pregnancy

There are a variety of determinants within the GU tract that can influence its health status. These include the constituents of the cervico-vaginal fluid (CVF), along with the resident microbiota. These aspects can, in turn, be altered by factors such as age and hormone status (Huggins and Preti, 1981).

1.2.1 Cervico-vaginal fluid

CVF contains transudate fluid and secretions from the Bartholin's glands (Huggins and Preti, 1981). Furthermore, CVF is composed of cervical mucus, endometrial and oviductal fluids and exfoliated cells, all of which vary in response to levels of hormones (Huggins and Preti, 1981). CVF has a role in the GU tract innate immune response to infection. A key role of CVF is to prevent access of microbes to the underlying cervicovaginal tissue. This is achieved by regularly discarding the mucus, along with sloughing of the superficial epithelial layer (Venkataraman et al., 2005). CVF also contains antimicrobials such as lysozyme and complement, alongside having a low pH and the presence of lactic acids (Valore et al., 2002;Cohen et al., 1984;Zegels et al., 2009). A number of cationic antimicrobial peptides have been isolated from CVF, including human beta-defensin 2 and cathelicidin. In total, 18 proteins have been isolated from CVF and identified as host defence proteins (Venkataraman et al., 2005). One study found that CVF collected from a small sample of donors allowed growth of *C. albicans*, but not GBS, in 3 of the 5 donors (Valore et al., 2002).

1.2.2 Resident microbiota

There are an estimated 10^8 bacteria for every gram of CVF (Delaney and Onderdonk, 2001). The resident microbiota can afford protection through colonisation resistance against incoming microbes. The dominant commensal microbes of the healthy vagina are *Lactobacillus* species, four of which predominate: *L. iners*, *L. crispatus*, *L. jensenii*, *L. gasseri* (Zhou et al., 2007;Ravel et al., 2011). Lactobacilli have been reported to use the glycogen harvested from routine sloughing of epithelial cells to generate lactic acid and maintain vaginal pH at 3.5-4.5. This serves to reduce the likelihood of colonisation by

pathogenic microorganisms (Nasioudis et al., 2015;Mirmonsef et al., 2014;Boris and Barbes, 2000). However, a more recent study found that the lactobacilli that most routinely colonise vaginal surfaces are unable to metabolise glycogen. Instead, these species metabolise the breakdown products of α-amylase digestion of glycogen (Nunn and Forney, 2016). Although vaginal tract α-amylase was previously assumed to be secreted by the host, it has been implied to be derived, in part, from other vaginal microbes (Nunn and Forney, 2016).

Oestrogen controls the rate of epithelial sloughing and hence the levels of free glycogen. As such, the vaginal microbiome is subject to change depending on ageing, the phase of the menstrual cycle or pregnancy (Farage and Maibach, 2006;Owen, 1975). There are reports of increasing *Lactobacillus* and decreasing non-*Lactobacillus* species as the menstrual cycle progresses, with highest levels of non-*Lactobacillus* species during menstruation (Eschenbach et al., 2000). This may be due to the microbial composition of the vaginal tract being most stable at peak oestrogen levels (Gajer et al., 2012).

Alongside acidification of the GU tract, lactobacilli can outcompete pathogenic microorganisms for host targets such as epithelial cells, and can release antimicrobial peptides (bacteriocins) (Cohen et al., 1984;Boris and Barbes, 2000). Another compound released by lactobacilli is hydrogen peroxide, which can induce oxidative stress in pathogenic bacteria (Tramer, 1966;Reid and Burton, 2002). Similar antimicrobial mechanisms have been described for lactobacilli in the gastrointestinal (GI) tract (Servin, 2004), which is important as the GI tract can act as a reservoir of microbes for GU tract colonisation. After lactobacilli, other frequent colonisers of the vaginal tract include *Peptococcus* species, *Bacteroides* species, *Staphylococcus epidermidis*, *Corynebacterium* species, *Peptostreptococcus* species, and *Eubacterium* species (Bartlett et al., 1977). Due to the continuity of the vaginal and cervical tracts, the cervical microbiome is largely homogenous to the vaginal microbiota (Smith et al., 2014;Huang et al., 2015).

1.2.3 Effects of pregnancy on the GU tract

During pregnancy, the mother undergoes a number of hormonal and metabolic changes to sustain the development of the foetus. In terms of metabolic changes, ghrelin, the 'hunger hormone', has receptors which are located in the placenta and reproductive

organs. Ghrelin levels increase up to the middle of a pregnancy, causing increased weight gain, high levels of fasting blood glucose and resistance to insulin (Fuglsang, 2008). These symptoms are also impacted by the maternal GI microbiome. A distinct difference has been found in GI microbes in each of the three trimesters, and this has been shown to affect the metabolism of the mother. Microbes from the stool of women in the third trimester conferred increased levels of fat, insulin resistance and inflammatory response in mice (Koren et al., 2012).

In terms of hormonal changes, high progesterone concentrations early in pregnancy favour secretion of Th2 rather than Th1 cytokines, and this is thought to be anti-inflammatory in order to maintain pregnancy (Kumar and Magon, 2012). There is also a steep rise in oestrogen concentrations, due in part to additional oestrogen secretion from the placenta (Nunn and Forney, 2016). High concentrations of oestrogen during pregnancy increase the levels of glycogen within the GU tract. As mentioned, glycogen harvested from exfoliated epithelial cells is used as a carbon source by vaginal microbes, and thus is hypothesised to support establishment of a *C. albicans* infection (Ma et al., 2012;Tarry et al., 2005;Dennerstein and Ellis, 2001).

The vaginal microbiota is considered stable during pregnancy due to consistent levels of hormones, with a reduction in overall microbial diversity. Pregnant women have a higher quantity of vaginal lactobacilli when compared to non-pregnant women, which largely consists of *L. crispatus* and *L. iners*, with a corresponding decrease in carriage of bacteria associated with BV (MacIntyre et al., 2015;Romero et al., 2014;Walther-Antonio et al., 2014). Factors contributing to this besides hormones are the reduced vaginal pH during pregnancy and the increase in vaginal secretions (Prince et al., 2014). As oestrogen levels drop after birth, levels of lactobacilli begin to decrease, underlining an important role for oestrogen in control of the vaginal microbiota (Romero et al., 2014;MacIntyre et al., 2015). Indeed, there is an increase in endometritis in postpartum women, correlating with a rise in BV-causing microorganisms, including *G. vaginalis*, *Bacteroides* spp. and GBS (Watts et al., 1989).

1.3 Group B *Streptococcus*

1.3.1 Genus *Streptococcus*

The first evidence of streptococci came from the 1683 drawings by van Leeuwenhoek of magnified matter scraped from his teeth. In 1879, Louis Pasteur isolated and identified streptococci from women suffering from puerperal fever, a postpartum condition which was often fatal. When the isolated streptococci were proven to cause the disease, they became the first identified infectious agent. The bacteria were named from the Greek '*strepto*' meaning twisted chain; and '*coccus*', due to the resemblance of their spherical appearance to berries. Streptococci are Gram positive (Figure 1-2) and facultatively anaerobic (Nobbs et al., 2009).

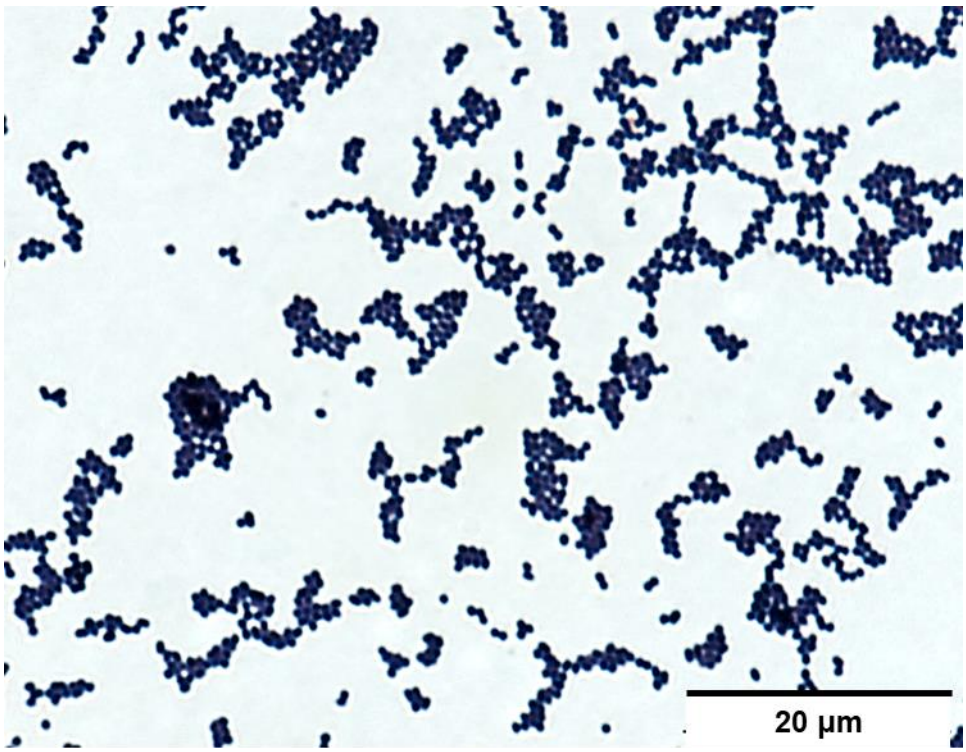

Figure 1-2 Gram stain of GBS strain NEM316.

Streptococci were traditionally classified, and in some cases named, due to Lancefield typing, whereby the composition of the streptococcal cell wall is used to categorise strains (Lancefield, 1933). In the case of groups A, B, C, E and G, this is based on polysaccharides, while for groups D and N this is based on teichoic acids, and group H is typed based on lipoteichoic acids (Rosan, 1973). Although this categorisation is effective for human pathogenic streptococci, the group antigens are in some species absent or

shared by multiple taxa (Nobbs et al., 2009). Alongside Lancefield typing, streptococci were also traditionally divided into groups based on their capacity to lyse red blood cells, known as haemolysis. Three categories were described: α, β or γ (Xu et al., 2014a). Streptococci are classed as α-haemolytic if an aura of green is formed when the bacteria are grown on blood agar. This arises due to oxidation of haemoglobin by hydrogen peroxide produced by the streptococci. This green colouration also reflects why some oral-colonising streptococci are referred to as 'viridans' streptococci (i.e. 'viridis' = Latin for 'green'). The pyogenic streptococci induce β-haemolysis on blood agar, which is characterised by a complete lysis of red blood cells and appears as a halo of yellow around bacterial colonies (Nobbs et al., 2009). The term γ-haemolysis is a bit of a misnomer as this is the category into which streptococci that are non-haemolytic are placed.

An alternative, and more recent, method of classifying streptococcal species is based on the 16S rRNA gene sequence. This divides streptococcal species into six groups (Figure 1-3). The pyogenic group includes known human and animal pathogens *S. pyogenes*, *S. agalactiae*, *S. uberis* and *S. dysgalactiae*. There is a wide range of diseases caused by pyogenic streptococci, including tonsillitis, toxic shock syndrome, necrotising fasciitis, neonatal sepsis and bovine mastitis (Edmond et al., 2012;Royster and Wagner, 2015;Cunningham, 2000). The mitis group of streptococci is made up almost exclusively of species which colonise the oral and nasopharyngeal cavities, and many of these species have been implicated in infective endocarditis, which is an inflammation in the lining of the heart (Barrau et al., 2004). Bovis group streptococci have been isolated from cases of meningitis and endocarditis (Amado et al., 2015;van Samkar et al., 2015), while mutans group streptococci are oral cavity colonisers and are aetiological agents of tooth decay (Kutsch, 2014). Anginosus and salivarius group streptococci are typically oral colonisers of humans and other animals (Nobbs et al., 2009).

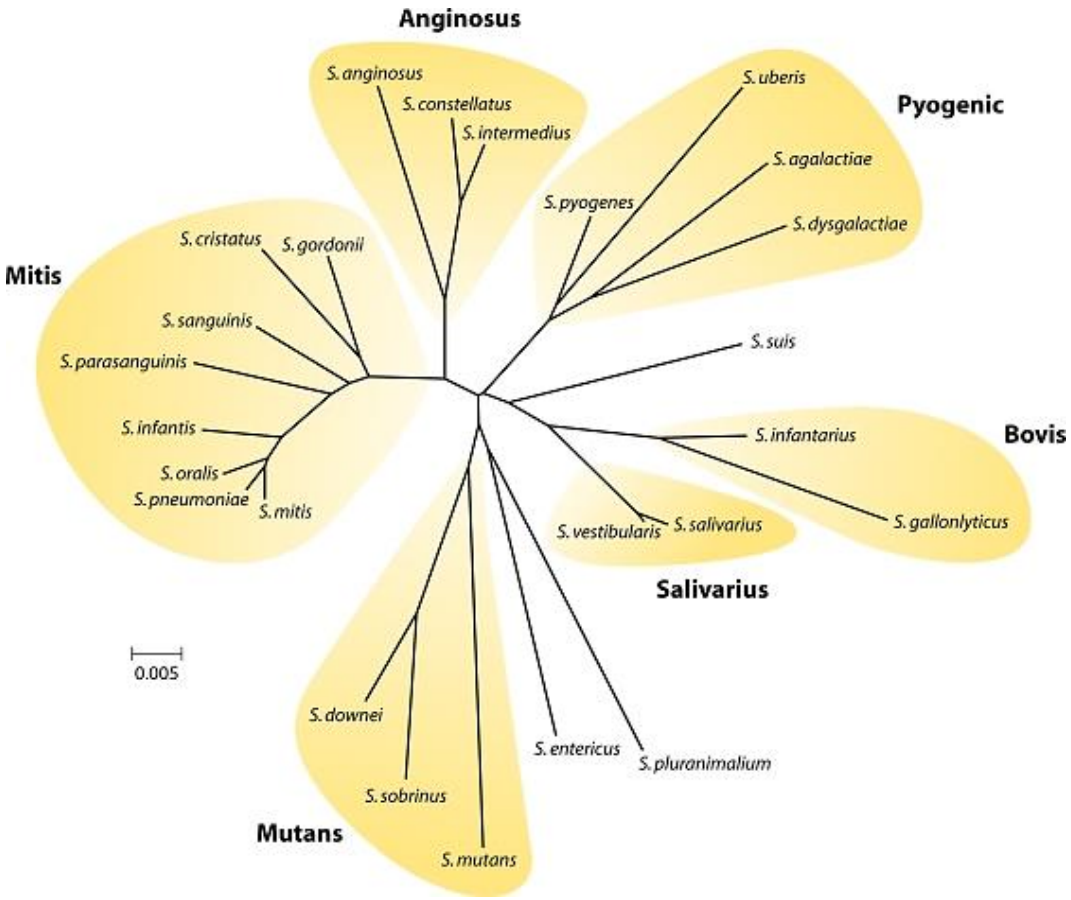

Figure 1-3 *Streptococcus* taxonomic relationships.

Classification of selected streptococcal species depending on 16S rRNA gene sequence. Taken from (Nobbs et al., 2009).

1.3.2 GBS overview

Streptococcus agalactiae is a group B, β-haemolytic *Streptococcus*; hence its alternate designation as Group B *Streptococcus* (GBS). GBS was first identified in 1887 as the causative agent of bovine mastitis (Bisharat et al., 2004;Lancefield and Hare, 1935). In 1935, GBS was isolated from human vaginal swabs and identified as the cause of fatal post-partum maternal infections. This was significant because all other serious streptococcal infections at that time were assumed to be caused by *S. pyogenes* (Group A *Streptococcus*, GAS) (Fry, 1938;Lancefield and Hare, 1935). GBS was identified as the predominant cause of neonatal infection in the 1960s and remains the foremost cause of neonatal sepsis and meningitis in Western countries (Eickhoff et al., 1964;Bisharat et al., 2004;Edmond et al., 2012). GBS is also increasingly a causative agent of disease in immunocompromised adults, among whom the disease rate is 4.1-7.2/100,000 people (Farley, 2001). The median age of GBS disease in non-pregnant adults is 63 years, and

the rate of disease among this population doubled between 1990 and 2007 (Skoff et al., 2009). One reason for this may be that cell-mediated immunity typically decreases with age, increasing the chance of infection (Edwards and Baker, 2005). Colonisation rates among the elderly are approximately 25%, similar to that of pregnant women (Edwards and Baker, 2005).

Alongside human infections, GBS causes disease in animals, particularly cattle and fish. Bovine mastitis, an inflammation of the mammary glands in cows, is a significant cause of disease affecting cattle. This costs the dairy industry in the US an estimated $2 billion annually (Wellenberg et al., 2002). The main causative organisms of bovine mastitis include *E. coli*, *Staphylococcus aureus* and GBS (Nair et al., 2005). GBS also causes disease in cats, dogs, aquatic mammals such as dolphins, crocodiles and even frogs (Brochet et al., 2006;Evans et al., 2008;Bishop et al., 2007;Amborski et al., 1983). A further area of particular concern is the incidence of GBS disease among fish farmed for human consumption, such as Nile tilapia. GBS is responsible for substantial morbidity and mortality, often causing meningoencephalitis (Evans et al., 2009;Pereira et al., 2010). Furthermore, consumption of fish was described by one study as a risk factor for human GBS colonisation with capsular serotypes commonly found to infect fish (Foxman et al., 2007).

1.3.3 GBS carriage and disease rate

In healthy adults, GBS has been found in 15-30% of GI or GU tracts, and the main reservoir of GBS is the GI tract (Jiang and Wessels, 2014;Le Doare and Heath, 2013;Carey et al., 2014). The overall rate of neonatal GBS disease in the UK is 0.5/1000 live births, according to the Royal College of Obstetricians and Gynaecologists (Hughes et al., 2012). However, the risk of neonatal disease rises to 2.3/1000 live births in the UK where an expectant mother has vaginal swabs that test positive for GBS. Furthermore, chance of colonisation in a future pregnancy, when the mother has been colonised in a previous pregnancy, is 38% (Hughes et al., 2012). There is limited data available on the rate of GBS disease in low and middle income countries; however, what data is available suggests that the mortality rate of neonatal GBS disease may be up to three times higher outside high income countries (Le Doare and Heath, 2013). This is reinforced by reports that disease rates may be as high as 3.06/1000 live births in parts of Africa

(Dagnew et al., 2012). Furthermore, an estimated 6-15% of all births are preterm, with the largest burden in Sub-Saharan Africa, leading to the deaths of approximately 4 million newborns annually. Of these preterm births, between 25-40% are thought to be due to bacterial infection, including GBS (Vornhagen et al., 2016).

Neonatal colonisation is 29 times more likely to occur if the mother is colonised (Boyer and Gotoff, 1985), and one study found that neonates had a three times higher chance of requiring intensive care if their mother was colonised compared to neonates born to un-colonised mothers, regardless of whether the neonates in question were culture-positive for GBS themselves (Brigtsen et al., 2015). A correlation has also been found between maternal anti-GBS antibody titres and infant health. Babies born to colonised mothers with anti-GBS antibody titres of more than 2 μg/mL sera more commonly suffered no infectious symptoms when compared to babies born to mothers with lower levels of antibody (Baker et al., 1981). This underlies the importance of maternal immune health, and provides evidence that a GBS vaccine could be effective in preventing neonatal disease.

1.3.4 Risk factors for GBS disease and colonisation

Limited information is available to determine the risk factors of GI tract colonisation; however, one study found that risk factors included black race, marijuana use, and an age of over 21 years (Meyn et al., 2009). Vaginal colonisation may be affected by a number of factors, including local hygiene, sexual practices, ethnicity, tampon or IUD use, obesity, or composition of the vaginal microbiota (Le Doare and Heath, 2013;Colbourn and Gilbert, 2007). Risk factors for vaginal carriage of GBS include a rise of vaginal pH above 4.5, experiencing vaginitis symptoms and unusual discharge (Leclair et al., 2010;Park et al., 2012). A number of microorganisms are thought to compete with GBS for colonisation in the vaginal niche. For example, *Bifidobacterium* exhibits antimicrobial activity against GBS *in vitro*, and *Lactobacillus* species can prevent the proliferation of GBS and subsequent adhesion to VECs (Ruiz et al., 2012;Zarate and Nader-Macias, 2006). Intravaginal challenge of mice with *Lactobacillus reuteri* prior to infection with GBS was found to influence the immune response generated against GBS. There was a reduction in influx of neutrophils, but a rise in activated macrophages and B cells, and this amounted to a protective effect on the host (De Gregorio et al., 2016). In

fact, an inverse relationship has been described between the presence of lactobacilli in the vaginal tract and the presence of GBS (Ronnqvist et al., 2006;Kubota et al., 2002). *Streptococcus salivarius* given as a probiotic was also shown to impair GBS using a mouse model of vaginal colonisation (Patras et al., 2015b).

1.3.5 GBS disease in neonates

The first signs of GBS infection in neonates include a blue appearance of the skin (cyanosis), jaundice, lethargy, fever, respiratory distress and seizure (Dong et al., 2017). There are two forms of GBS neonatal disease, classified according to the onset of these symptoms. Early onset disease (EOD) is an infection which presents by 6 days old (Baker and Barrett, 1974;Weisner et al., 2004;Zaleznik et al., 2000) and this accounts for 60-70% of all neonatal GBS disease in developed countries (Le Doare and Heath, 2013). EOD typically presents as pneumonia or sepsis (Heath et al., 2004). Sepsis results from prolonged survival of GBS in the blood and significant cytokine induction (Berner et al., 2001), while pneumonia results from aspiration of GBS-infected material during birth, followed by subsequent invasion and damage of lung tissues (Doran et al., 2002). EOD requires the mother to be carrying GBS in the GU tract, leading to vertical transmission *in utero* or during parturition as the predominant mode of transmission (Baker and Barrett, 1974;Weisner et al., 2004;Colbourn and Gilbert, 2007). Approximately 20-30% of pregnant women carry GBS vaginally in Western countries, with 50% of their children becoming colonised, and 1% progressing to invasive disease (Le Doare and Heath, 2013;Hughes et al., 2012). One study claimed that risk of transmission from the maternal GU tract to the neonate was as high as 70% (Bodaszewska-Lubas et al., 2013).

Late onset disease (LOD) manifests 7-89 days after birth (Hughes et al., 2012;Weisner et al., 2004;Harrison et al., 1998). Infants may become infected nosocomially or from community sources; however, the mother may still be the primary source of transmission (Weisner et al., 2004;Edmond et al., 2012). Meningitis has been observed in over 40% of LOD cases (Heath et al., 2004;Weisner et al., 2004), and contributes to substantial neurological sequelae. Around half of children surviving GBS meningitis had some form of disability such as sight or hearing loss (Bedford et al., 2001;Libster et al., 2012). Meningitis results from GBS first surviving within the bloodstream and then crossing the blood brain barrier (BBB), leading to induction of cerebral oedema, cerebral

ischaemia, increased intercranial pressure and inflammation, predominantly mediated by neutrophils (Doran et al., 2016).

1.3.6 GBS disease in adults

GBS is capable of causing disease in immunocompromised adults, although the disease burden is largely among those suffering from a chronic illness, such as diabetes or cancer, and is often nosocomially acquired (Fujita et al., 2015;Farley, 2001). In fact, 20-25% of GBS infections of non-pregnant adults are in patients with diabetes (Farley, 2001). The adult form of disease typically presents as non-specific bacteraemia, or as infections of the skin and soft tissue, and only rarely presents as meningitis or endocarditis (Fujita et al., 2015;Scully et al., 1987a;Sambola et al., 2002;Ross, 1984).

1.3.7 GBS classification and disease

One major system used for the classification of GBS is based on expression of different types of capsular polysaccharide (CPS). There are ten serotypes of CPS (Ia, Ib and II-IX) that are antigenically and structurally distinct, and their distribution seems to be uniform worldwide (Alkuwaity et al., 2012;Edmond et al., 2012). Four CPS serotypes are associated with most disease: Ia, II, III and V (Zhang et al., 2006;Jiang et al., 2008;Farley, 2001). Serotypes Ia, III and V are the cause of around 80% of EOD and 92% of LOD disease (Alkuwaity et al., 2012;Weisner et al., 2004;Zaleznik et al., 2000). CPS serotype III is associated with a higher incidence of infant meningitis (Baker and Barrett, 1974;Weisner et al., 2004;Harrison et al., 1998).

GBS strains can also be classified according to sequence type (ST); otherwise known as clonal complex (CC). Multilocus sequence typing comprises the sequencing of fragments around 500 bp in length of 7 housekeeping genes in order to group bacterial strains and investigate their population dynamics (Jones et al., 2003). Human pathogenic GBS strains belong to six CCs: CC-1, -10, -17, -19, -23, -26 (Jones et al., 2003;Da Cunha et al., 2014), and one study found that just four CCs (CC-1, -17, -19, and -23) accounted for more than half of all isolated GBS (Jones et al., 2006). Of these, CC-17 was found to be significantly more associated with invasive neonatal disease than the others. All GBS

isolates which were identified as CC-17 were also CPS III (Jones et al., 2006). EOD is significantly associated with CC-23 of CPS Ia, as well as CC-17 of CPS III (Bohnsack et al., 2008;Martins et al., 2007;Martins et al., 2017). More than 80% of LOD cases are caused by CC-17 strains, which are also highly associated with meningitis (Poyart et al., 2008;Martins et al., 2007;Tazi et al., 2010). CC-17 is responsible for a greater proportion of neonatal GBS disease than might be expected based on maternal colonisation by this CC (Jones et al., 2006;Martins et al., 2017).

1.3.8 Molecular basis of GBS pathogenesis

1.3.8.1 Adhesion to epithelial tissues

Adhesion to host surfaces is an important first step in the pathogenesis of GBS disease. Consequently, GBS possesses many proteins at the cell surface which act as adhesins, facilitating attachment and colonisation of host mucosae (Figure 1-4). Some of these adhesins are able to target receptors directly expressed on the host cells, while many others seemingly utilise an ECM bridging molecule such as fibronectin, fibrinogen or laminin.

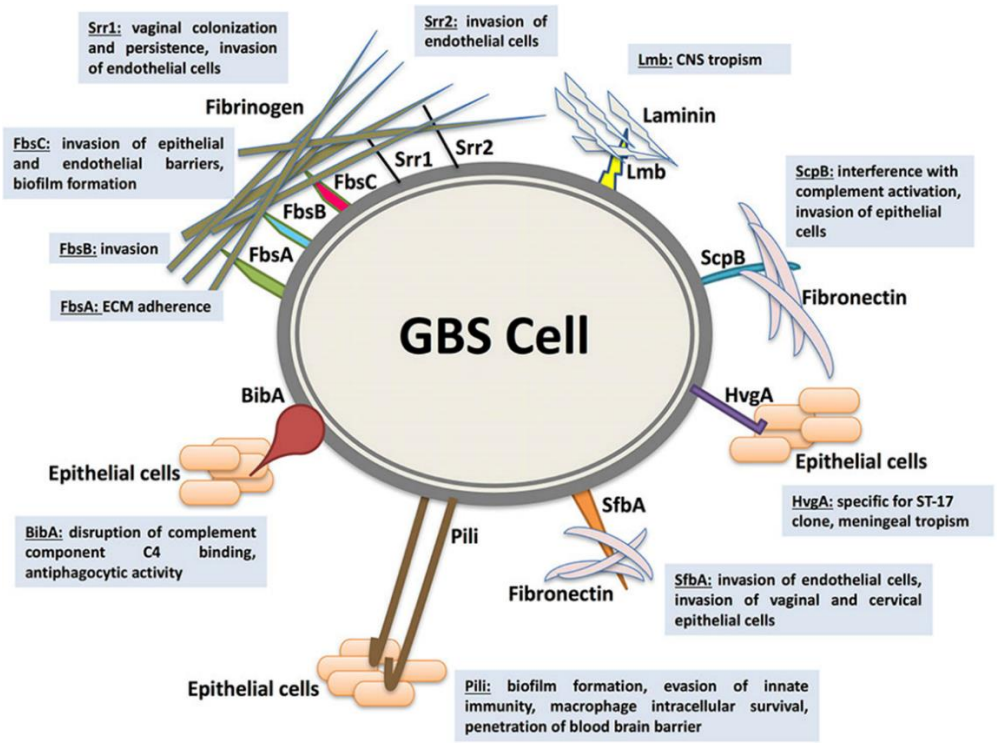

Figure 1-4 Schematic of GBS cell surface proteins.

Taken from (Shabayek and Spellerberg, 2018).

Alpha C protein is a GBS cell surface protein which contains long tandem repeats. Alpha C protein of GBS has been shown to bind to and facilitate invasion of GBS into cervical epithelial cells *in vitro* (Bolduc et al., 2002). WT GBS invaded at 3-fold greater numbers than the null mutant, and was transcytosed across cervical cells at 5-fold greater numbers (Bolduc et al., 2002). A neonatal mouse model of infection showed that an alpha C protein null mutant was 7-fold reduced in virulence compared to WT, and led to increased host survival (Li et al., 1997).

Pili are long, thin structures extending away from the bacterial cell surface and are important for adhesion to host cells. The protein subunits and enzymes required for pilus biogenesis are encoded by loci known as pilus islands (PI), which can be separated into variants PI-1 and PI-2, with PI-2 further subdivided into PI-2a and b (Rosini et al., 2006). GBS pili are composed of three proteins: a major backbone subunit, an accessory anchor subunit and a tip-associated adhesin subunit. Both pilus types 1 and 2a mediate GBS adhesion to HeLa and A459 cells (Dramsi et al., 2006;Pezzicoli et al., 2008), and promote GBS survival in a mouse model of vaginal infection (Sheen et al., 2011). Pilus type 2a, but not other pili, is also involved in biofilm formation (Rinaudo et al., 2010).

FbsC is a cell surface protein of GBS which binds to fibrinogen in a dose-dependent manner, with fibrinogen binding by GBS ablated by the deletion of FbsC (Buscetta et al., 2014). Deletion of FbsC significantly decreased adherence of GBS to endothelial brain cells, intestinal and lung epithelial cell lines, as well as reducing biofilm formation (Buscetta et al., 2014). Mice were found to be protected from killing by GBS after inoculation with FbsC, and mice had an increased survival and decreased GBS burden when challenged with an FbsC null mutant (Buscetta et al., 2014). Similarly, adhesin BsaB has been shown to be important for GBS binding fibronectin, VECs and cervical epithelial cells *in vitro*. When constitutively expressed on the surface of Gram positive surrogate host *Lactococcus lactis*, BsaB conferred the ability to bind ECM components fibronectin and laminin, as well as VECs and cervical epithelial cells. BsaB also enabled biofilm formation in *L. lactis* (Jiang and Wessels, 2014).

Serine-rich repeat (SRR) proteins are cell surface adhesins which are highly glycosylated, and are found in many Gram positive bacteria, where they have been described to contribute to adhesion to host cells (Stinson et al., 2003;Siboo et al., 2005). SRR protein-1 (Srr-1) of GBS has been shown to bind fibrinogen and promote vaginal and cervical

colonisation (Wang et al., 2014;Sheen et al., 2011). A mutant GBS strain deficient in Srr-1 was significantly reduced in binding VECs, endocervical and ectocervical cells, with WT function restored by complementation (Sheen et al., 2011). In a mouse model of vaginal infection, GBS deficient in Srr-1 was impaired in persistence (Sheen et al., 2011). The only receptor-adhesin pair definitively described for GBS is that of Srr-1 and cytokeratin 4 (Sheen et al., 2011).

One of the reasons that GBS CC-17 is considered more virulent than other CCs is due to expression of hypervirulent adhesin HvgA, which is specific to CC-17 strains (Tazi et al., 2010). Expression of HvgA allowed increased adhesion of GBS to intestinal epithelial cells, choroid plexus cells of the brain, and cells of the BBB. Moreover, expression of HvgA in normally non-adherent GBS strains facilitated adhesion to these cell types (Tazi et al., 2010).

Alongside surface proteins, adherence of GBS to adult oral epithelial, foetal or embryonic cells was ablated in conditions where lipoteichoic acid (LTA) was not expressed on the GBS cell surface. In addition to this, pre-incubation of epithelium with purified LTA prevented adhesion of GBS to these tissues (Nealon and Mattingly, 1984), and a further study replicated this effect with neonatal buccal epithelial cells and VECs (Teti et al., 1987). Expression of LTA on the surface of GBS is also crucial for penetration of the BBB and subsequent establishment of meningitis (Doran et al., 2005). Mice challenged with a GBS LTA mutant survived in significantly higher numbers than those challenged with WT GBS (Doran et al., 2005).

1.3.8.2 Crossing the blood brain barrier

In addition to those mentioned above, proteins implicated in enabling GBS to cross the BBB include PilA and PilB (Maisey et al., 2007), Lmb (Tenenbaum et al., 2007), SfbA (Mu et al., 2014) and FbsA (Tenenbaum et al., 2005). GBS Δ*pilA* was 60% reduced in adherence but not invasion to human brain microvascular endothelial cells (HBMECs), while a Δ*pilB* strain was 40% reduced in internalisation but not adherence, suggesting a role for PilA in preliminary binding to the BBB, while PilB facilitates invasion (Maisey et al., 2007). WT function was restored by complementation. Supporting this, when *pilA*

and *pilB* were heterologously expressed in *L. lactis*, PilA increased attachment to HBMEC by 20-fold, while PilB enhanced invasion by 55-fold (Maisey et al., 2007).

Lmb, laminin-binding protein, plays a role in invasion of GBS across the BBB. Strains with Lmb deleted were impaired in HBMEC invasion by around 65%, but unaffected in adhesion (Tenenbaum et al., 2007). In competitive binding experiments, invasion of HBMEC by GBS was reduced by around 45% when in the presence of either excess purified recombinant Lmb or antibodies directed at the binding region of Lmb (Tenenbaum et al., 2007).

SfbA is a fibronectin binding protein. In the absence of SfbA, GBS is 4-fold reduced in binding fibronectin (Mu et al., 2014). When SfbA was heterologously expressed in *L. lactis*, fibronectin binding was increased by 2-fold. GBS Δ*sfbA* is significantly reduced in invasion of HBMEC cells, with this effect reversed by complementation, as well as VECs and cervical cells. However, there was no significant difference between the HBMEC adherent colony forming units (CFU) of the Δ*sfbA* strain compared to WT (Mu et al., 2014). SfbA has further been hypothesised to be involved in the pathogenesis of meningitis, as an *in vivo* mouse model found that mice infected with GBS *ΔsfbA* either did not have neutrophil infiltration and thickening of the meningeal layer (characteristics of GBS meningitis), or this was much reduced compared to WT-infected mice. This was attributed to the significantly lower number of Δ*sfbA* bacteria observed to penetrate the BBB compared to WT (Mu et al., 2014).

FbsA is a fibrinogen binding protein. Attachment and invasion of HBMEC by GBS Δ*fbsA* was around 90% decreased when compared against WT, and this was reversed by complementation (Tenenbaum et al., 2005). When FbsA was heterologously expressed in *L. lactis*, there was a significant increase in attachment to but not invasion of HBMEC when compared against the empty vector control. This suggests that FbsA plays an important role in initial attachment to HBMEC but is not an invasin (Tenenbaum et al., 2005). Adherence and invasion of HBMEC was competitively inhibited in a dose-dependent manner by increasing concentrations of monoclonal antibodies directed against the binding region of FbsA (Tenenbaum et al., 2005).

Another suggested mechanism by which GBS may cross the BBB is by binding plasmin and plasminogen to the GBS surface (Magalhaes et al., 2013). Although the GBS proteins

involved in binding plasminogen have not been confirmed, they are hypothesised to be the GBS enolase and GAPDH (Magalhaes et al., 2013).

1.3.8.3 Uterine infection

As mentioned, EOD by GBS can be initiated following infection of the foetus *in utero*. Such infection can also lead to stillbirth, chorioamnionitis or preterm birth (Nan et al., 2015;Muller et al., 2006), and requires that GBS ascends from the vaginal vault during pregnancy to cross the cervix and enter the uterus. Hyaluronic acid is a constituent of the host ECM and plays a critical role in epithelial barrier integrity. GBS expresses a hyaluronidase, HylB, which degrades hyaluronic acid and this has been implicated to aid in ascending GBS infection (Vornhagen et al., 2016). Clinical isolates of GBS from women who went into preterm birth exhibited higher levels of hyaluronidase activity than was observed in commensal GBS strains, and this was also the case for strains isolated from amniotic fluid or the blood of neonates (Vornhagen et al., 2016). Significantly fewer pregnant mice developed ascending infection when challenged with a Δ*hylB* mutant compared to WT, with fewer pups experiencing an adverse outcome and a lower rate of preterm birth (Vornhagen et al., 2016). Although there were similar levels of vaginal colonisation, bacterial load in the uterine horns was significantly lower in the Δ*hylB*-infected pregnant mice (Vornhagen et al., 2016). Degradation of hyaluronic acid into its component disaccharides has been shown to block TLR2 and TLR4, thus circumnavigating the host immune response (Kolar et al., 2015), and this was supported by a greater proinflammatory immune response to Δ*hylB* than WT GBS (Vornhagen et al., 2016).

Another virulence factor of GBS, β-haemolysin/cytolysin (β-H/C) is involved in promotion of GBS disease following ascending uterine infection. There was an ablation of stillbirth or preterm birth in pregnant mice challenged with a β-H/C deficient strain compared to WT, although both strains colonised and ascended (Randis et al., 2014). β-H/C induced inflammation in the placenta and GBS invasion of the foetus, particularly into the lungs and liver (Randis et al., 2014). Deletion of the two-component regulator of virulence CovR resulted in a hyper-haemolytic strain and increased the rate of preterm birth (Whidbey et al., 2015). β-H/C expression was also associated with increased foetal death in pregnant mice (Whidbey et al., 2015). This was further supported in a model of non-

human primate pregnancy, in which β-H/C-expressing GBS strains were associated with preterm birth, amniotic infection and foetal sepsis (Boldenow et al., 2016). In this model, β-H/C induced neutrophil cell death and facilitated evasion of neutrophil extracellular traps (NETs), which allowed GBS to subsequently cause foetal infection (Boldenow et al., 2016).

1.3.8.4 Evasion of host defences

In order to successfully colonise and establish an infection, GBS must avoid clearance by host immune defences. One surface determinant that enables GBS to achieve this is the capsule. The GBS capsule is largely composed of monosaccharides of glucose, galactose and *N*-acetylglucosamine with side chains of sialic acid (Cieslewicz et al., 2005). The sialic acid component of CPS mimics host glycoproteins and glycolipids, masking GBS from the immune response (Cieslewicz et al., 2005). The capsule of GBS is resistant to complement-mediated killing by leukocytes, effectively evading the immune system (Marques et al., 1992). The amount of capsule expressed, specifically sialic acid within the capsule, was shown to be inversely proportional to the ability of complement C3 to bind the bacteria (Takahashi et al., 1999;Marques et al., 1992). Loss of capsule by GBS led to decreased virulence in a rat model of infection (Wessels et al., 1989).

Other GBS virulence factors which prevent clearance of GBS from the host include C protein, C5a peptidase and CspA. C protein contains domains which bind secretory IgA (S-IgA) to sequester the antibody and prevent clearance (Jerlstrom et al., 1996). C5a peptidase degrades C5a, a potent neutrophil chemoattractant, allowing persistence of GBS (Bohnsack et al., 1997). CspA is a cell surface serine protease with some homology to C5a peptidase. CspA cleaves fibrinogen, the breakdown products of which were hypothesised to encourage GBS aggregation or possibly to coat GBS, thus masking the bacteria from the immune system (Harris et al., 2003). A CspA deficient strain was ten-fold less virulent in a neonatal rat model of infection, and was more susceptible to neutrophil-mediated killing (Harris et al., 2003).

Additionally, β-H/C has been shown to contribute to the survival of GBS within the phagolysosome of phagocytic cells. GBS strains deficient in β-H/C were more rapidly cleared from the blood stream of mice than WT, and were more susceptible to oxidative

killing. This was related to the ability of GBS to induce cytolysis and apoptosis of phagocytic cells via β-H/C (Liu et al., 2004b). Protection against oxidative killing by macrophages is further mediated by expression of a superoxide dismutase, SodA (Poyart et al., 2001). A SodA mutant was shown to be significantly more vulnerable to oxidative stress when exposed to hydrogen peroxide or when grown with macrophages. Furthermore, there was a significant reduction in the ability of the SodA mutant to survive in the blood or brain of mice, although similar levels of bacteria were recovered from the liver and spleen as for WT (Poyart et al., 2001).

1.3.9 GBS control measures

1.3.9.1 Intrapartum antibiotic prophylaxis (IAP)

Given the risk of maternal vertical transmission of GBS, a number of Western countries screen for GBS GU tract colonisation at 35-37 weeks of pregnancy, and offer intrapartum antibiotics as necessary (Schrag et al., 2002). Randomised controlled trials treating pregnant mothers who displayed likely risk factors for GBS colonisation with ampicillin and penicillin were carried out in the 1970s and 1980s; however, IAP was not implemented until the late 1990s due to issues with identifying which pregnant women should receive IAP (Schrag and Verani, 2013). Antibiotics prescribed around birth have reduced the rate of EOD in the US by 65%, yet they have not had an effect on the rate of LOD (Schrag et al., 2000). Unfortunately, GBS-related stillbirths are also unaffected by the intervention of IAP (Nan et al., 2015). A systematic review found that the rate of stillbirths was difficult to identify due to a lack of consistency in reporting (Nan et al., 2015).

A further issue with IAP is that, although the CDC has released clear guidelines for US health workers, these guidelines are not always followed. IAP usually consists of either intravenous penicillin or ampicillin, with cefazolin recommended as an appropriate alternative for women with penicillin allergies (Schrag and Verani, 2013). One study assessing the effectiveness of IAP found that less than a third of cases in which mothers had a risk factor which would qualify them for IAP resulted in provision of antibiotics (5 of 16) (Darlow et al., 2016). A further study found that 40% of IAP cases reviewed received an inappropriate antibiotic (Bienenfeld et al., 2016), while another found that 55% of women reporting a penicillin allergy were given an inappropriate antibiotic as

substitute (Briody et al., 2016). In Italy, women delivering pre-term were found to be less likely to receive IAP, while 817 women were given IAP against recommendations (i.e. when culture-negative for GBS) (Berardi et al., 2017). Furthermore, despite similar levels in IAP compliance in the US, the rate of GBS EOD is twice as high in preterm neonates as among those born at term (Schrag and Verani, 2013).

There are understandable concerns about widespread antibiotic use and resulting resistance. There have been reports that IAP increases colonisation by potentially harmful Enterobacteriaceae in the infant once born, as the microbes which are normally acquired by passage through the birth canal and make up the infant's initial microbiota are less diverse (Mazzola et al., 2016;Aloisio et al., 2014). Diversity of intestinal microbes is important in early life, and plays a role in development of intestinal epithelium and associated lymphoid tissue (Gritz and Bhandari, 2015). IAP also increases the mother's risk of diarrhoea, fungal infections and antibiotic resistance, while affecting the child by increasing the risk of *E. coli* infections, thrush and allergic sensitivity. As such, both mother and child are kept in for observation for 48 hours after birth, which is costly (Hanson et al., 2014).

On average, countries which implement IAP have lower rates of neonatal GBS disease than countries which do not use IAP. However, this may be skewed by including poorer countries in which pregnant mothers have less access to regular medical treatment (Edmond et al., 2012). IAP has also only been described to be cost-effective in countries where there are higher rates of GBS recto-vaginal colonisation (Albright et al., 2017). Intravenous antibiotic treatment is not possible in resource poor settings, and neither is intrapartum GBS screening, as this involves resources and access to care facilities that are less than realistic in poorer countries and rural locations (Schrag and Verani, 2013;Nishihara et al., 2017).

1.3.9.2 Vaccines

Given the issues associated with IAP, vaccination against GBS is an attractive alternative. CPS Ia, Ib, II, III and V are the five most common serotypes associated with GBS disease (Le Doare and Heath, 2013) and as such, these are the serotypes that have predominantly been used as targets in vaccine studies against GBS (Nuccitelli et al.,

2015). Native CPS alone was found to be of insufficient immunogenicity, but second generation vaccines in which the polysaccharides are conjugated to a strong immunogen such as tetanus toxoid have been shown to trigger a robust anti-CPS antibody response (Paoletti et al., 1994;Leroux-Roels et al., 2016;Madhi et al., 2017). Nonetheless, immune responses are serotype-specific, meaning that multivalent vaccines are required to obtain sufficient coverage. Furthermore, capsular switching of GBS serotype III CC-17 to serotype IV has been described (Bellais et al., 2012;Teatero et al., 2014;Meehan et al., 2014), which could further reduce the efficacy of any CPS-based vaccine. No licensed vaccine is available as yet; however, serum containing high levels of antibodies against CPS among pregnant mothers has been described as protective against GBS invasive neonatal disease (Baker and Kasper, 1976). Modelling has estimated that a vaccine would be much more cost-effective and preventative than alternative strategies, preventing between 30-54% of neonatal infections, depending on efficacy against the most common CPS. This compares to 10% prevention, as observed with risk factor-based IAP (Kim et al., 2014). Despite cost-efficacy, vaccine compliance has been estimated to be required at high levels (~90%) before a reduction in disease rates would be observed at similar levels to those seen for screening/IAP (Kim et al., 2014).

In response to the potential issues of a GBS vaccine based on CPS immunogens only, attempts are underway to identify highly protective protein antigens. One study screened GBS proteins for immunogenicity in mice and found that four antigens were sufficient for a significant rise in survival in progeny mice. These were Sag0032 (Sip protein), part of the core GBS genome, and Sag1408, Sag0645, and Sag0649, all of which encode cell surface-expressed pilus subunits (Maione et al., 2005;Lauer et al., 2005). Between 59-100% of neonatal mice were protected from lethal infection when challenged with 12 different GBS strains following maternal immunisation with all four proteins. Furthermore, opsonophagocytosis and neutrophil-mediated killing of GBS was enhanced following vaccination with these antigens (Maione et al., 2005). Protective antibodies have been generated to a conserved region of PI-2a in mice, and this evidence is being used to further the development of vaccines against pili (Nuccitelli et al., 2011).

Other proteins of GBS have also been considered as vaccine targets. Pullulanase is a glycoside hydrolase involved in α-glucan degradation and attachment to cervical epithelial cells. Pullulanase is immunogenic, and serum from animals immunised with

pullulanase prevented the activity of the enzyme and altered the ability of GBS to attach to cervical tissue (Gourlay et al., 2009). SRR protein SAN_1485 has also been found to be a protective cell surface antigen (Doro et al., 2009). MinervaX have announced positive results from a phase I clinical trial with a vaccine directed against GBS proteins Alpha C and Rib, which resulted in a 30-fold increase in maternal antibodies to these proteins (Minervax, 2017).

1.4 *Candida albicans*

1.4.1 Overview

Only 600 of an estimated 611,000 fungal species are human pathogens, including those that cause relatively non-threatening superficial and cutaneous infections (Mayer et al., 2013;Brown et al., 2012). Yeasts of the genus *Candida* can cause systemic infections with fatality rates up to 50%, and are the fourth most common cause of systemic nosocomial infections in the US, although these deaths are largely among immunocompromised individuals (Pfaller and Diekema, 2010;Pfaller and Diekema, 2007;Zaas et al., 2010). Age and HIV infection are key risk factors because they impair the adaptive immune response, thus predisposing individuals to *Candida* infections (Pfaller and Diekema, 2010).

Candida albicans is the principal cause of fungal infections affecting humans; however, *C. albicans* is also a common human commensal (Harriott and Noverr, 2011). *C. albicans* is responsible for approximately 90-99% of all mucosal *Candida* infections, and is able to infect a broad range of host tissues, causing disease even in otherwise healthy people (Harriott and Noverr, 2011;Zaas et al., 2010;Pfaller and Diekema, 2010).

1.4.2 *C. albicans* polymorphism

C. albicans is a pleomorphic yeast. The two most important morphological forms in terms of colonisation and pathogenesis are the budding yeast form (also known as blastospores) and the elongated (filamentous) hyphal form (Fanning and Mitchell, 2012) (Figure 1-5).

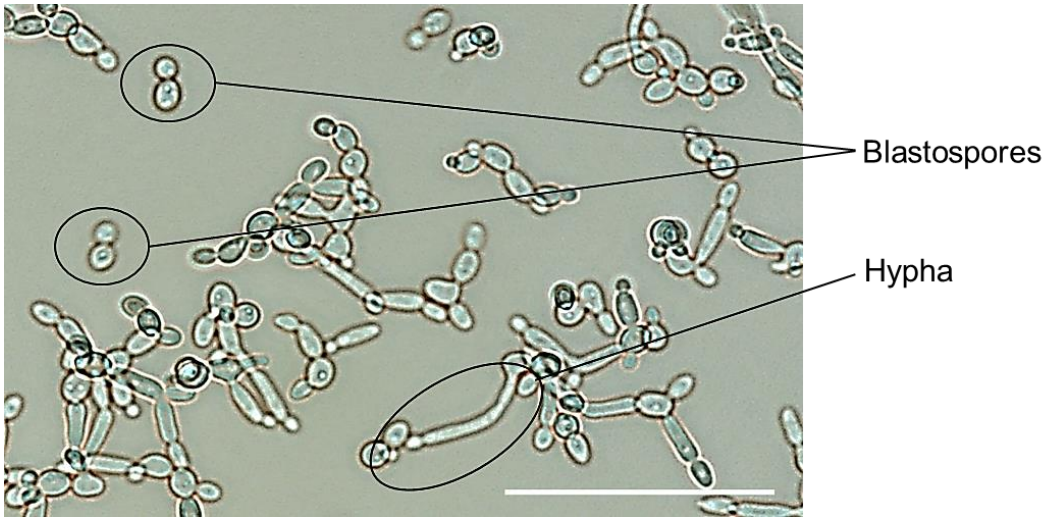

Figure 1-5 Polymorphism of *C. albicans*.

Brightfield microscopy with blastospores and hypha labelled. Scale, 50 µm.

To be pathogenic, *C. albicans* must be in its hyphal form, which allows invasion of host tissues (Monif and Carson, 1998). A pH below 6 triggers yeast growth, whilst a pH above 7 triggers hyphal growth (Mayer et al., 2013). Additionally, temperatures similar to that of the human body (~37 °C), supplementation of media with *N*-acetyl-D-glucosamine (GlcNAc) or serum, and 5% CO_2 stimulate hyphae formation (Sudbery, 2011;Shapiro et al., 2009;Mardon et al., 1969;Simonetti et al., 1974;Taschdjian et al., 1960). Quorum sensing molecules of *C. albicans* also affect yeast to hypha transition. Farnesol inhibits hyphae formation (Hornby et al., 2001), while tyrosol promotes filamentation (Chen et al., 2004). These molecules are believed to be continuously secreted in response to environmental conditions to stimulate or limit growth and biofilm formation by *C. albicans* (Alem et al., 2006;Ramage et al., 2002). The pleomorphic nature of *C. albicans* is essential for colonisation and adherence to host tissues (Shirtliff et al., 2009). This was demonstrated through the use of a *C. albicans* strain which formed hyphae or blastospores under controllable conditions. Mice infected with this strain under conditions allowing hyphae formation were susceptible to infection, while mice under conditions predisposing to blastopores were not, despite equal loads of *C. albicans* (Saville et al., 2003).

1.4.3 *C. albicans* carriage and disease

1.4.3.1 Carriage

C. albicans is estimated to be present in the oral cavity of 75% of people (Mayer et al., 2013). *C. albicans* is also a commensal of the female GU tract, from which it is frequently isolated (Monif and Carson, 1998). It is the most commonly recovered yeast from the vaginal tract (Ng et al., 1998), with one study reporting *C. albicans* accounting for 83.5% of *Candida* species from vaginal swabs taken from pregnant women (Masri et al., 2015). Furthermore, *C. albicans* has been isolated from the vaginal tracts of around 30% of healthy, non-pregnant women without symptoms (Akimoto-Gunther et al., 2016;Beigi et al., 2004).

1.4.3.2 Candidemia

C. albicans causes 40-70% of candidemia (*Candida* within the blood stream) cases (Harriott and Noverr, 2011;Zaas et al., 2010;Pfaller and Diekema, 2010). The incidence of candidemia has been shown to be rising in both the US and France, where it stands at approximately 5.6 and 3.6 candidemia-related hospitalisations per 100,000 people, respectively, although this rate is higher among ICU patients (Zilberberg et al., 2008;Bitar et al., 2014). Candidemia can have fatality rates of 30-60%, although this is predominantly amongst immunocompromised individuals (Flevari et al., 2013). Risk factors for candidemia include advanced age, catheter use, parenteral nutrition and broad-spectrum antibiotic use (Hirano et al., 2015;Luzzati et al., 2013). Other causative agents of candidemia include *C. parapsilosis*, *C. tropicalis*, *C. glabrata* and *C. krusei* and, together with *C. albicans*, these strains account for over 90% of all candidemia cases (Guinea, 2014;Falagas et al., 2010).

1.4.3.3 Thrush

C. albicans typically behaves as a commensal microorganism within the oral cavity, but can cause oral disease in immunocompromised individuals, for example the elderly or people who suffer from HIV. A further risk factor is wearing dentures (Pappas et al., 2009), and among denture wearers smoking and diabetes has been correlated with a higher incidence and burden of *C. albicans* colonisation (Abu-Elteen and Abu-Alteen,

1998). Symptoms of oral thrush include the formation of white plaques on the oral mucosa, a sensation of burning, changes in taste, and bleeding at the site of the plaques (Millsop and Fazel, 2016).

It is estimated that around 75% of women will suffer from vaginal thrush, also known as vulvovaginal candidiasis (VVC), at least once in their lifetime, and 40-50% will suffer one or more subsequent infections (Harriott and Noverr, 2011;Sobel, 2007;Harriott et al., 2010). Symptoms typically present as itching, soreness, pain during intercourse and odourless, white discharge (Sobel, 2015). Risk factors for VVC include oral contraception, hormone therapy, antibiotics, diabetes, and pregnancy (Harriott and Noverr, 2011;Sobel, 2007;Andes et al., 2004). *C. albicans* is the most common *Candida* species involved in colonisation and infection of the vaginal tract (Pfaller and Diekema, 2007). *C. albicans* colonisation of the vaginal tract is enhanced by an oestrogen environment, and *C. albicans* can travel to the vaginal tract via the lower GI tract, similar to GBS (Sobel, 2007). Amongst women with no symptoms of VVC, 20-30% will still be colonised with *C. albicans* (Achkar and Fries, 2010).

1.4.3.4 Neonatal *C. albicans* infection

C. albicans can cause neonatal sepsis and meningitis, although the affected infants are most commonly premature with a low birthweight (Lee et al., 1998;Botero-Calderon et al., 2015;Arsenault and Bliss, 2015). Although there is variation between studies, over 60% of neonatal *Candida* infections are caused by *C. albicans* (Lee et al., 1998;Saiman et al., 2000;Lovero et al., 2016;Botero-Calderon et al., 2015;Fernandez et al., 2000), with *C. parapsilosis* being the second most common causative species (Saiman et al., 2000;Lovero et al., 2016;Botero-Calderon et al., 2015). Disease has a high mortality rate and, as with GBS, meningitis survivors often develop neurological defects, such as sight or hearing loss, cognitive deficits or cerebral palsy (Lee et al., 1998;Saiman et al., 2000;Lovero et al., 2016;Botero-Calderon et al., 2015;Arsenault and Bliss, 2015;Benjamin et al., 2006;Benjamin et al., 2003). Risk factors for neonatal *Candida* infections are low birthweight, prematurity, use of a central venous catheter and third generation antibiotic prescription (Lovero et al., 2016;Botero-Calderon et al., 2015). One study suggested that *Candida* may be vertically transmitted from the mother during vaginal birth (Botero-Calderon et al., 2015). Furthermore, (Saiman et al., 2000) found

that 43% of infants with candidemia had *Candida*-colonised GI tracts, with Benjamin et al. (2003) finding that only half of cases were colonised nosocomially, suggesting another source of transmission (Benjamin et al., 2003). Thus, alike to GBS, *C. albicans* can be transmitted to a neonate from a colonised mother or via horizontal transmission.

1.4.4 Molecular basis for *C. albicans* pathogenesis and colonisation

1.4.4.1 Adhesion

C. albicans expresses a number of cell wall adhesins that have been associated with attachment to mucosal tissues. These include Als1, Eap1 and Tdh1. Als1 is a *C. albicans* protein with a high sequence identity to alpha-agglutinin, an adhesin of *S. cerevisiae* (Hoyer et al., 1995). A *C. albicans* Als1 null mutant was 20% less able to adhere to human umbilical vein endothelial cells and to cause damage to reconstituted oral epithelium than the WT (Zhao et al., 2004b). Expression of Als1 on the cell surface of *S. cerevisiae* also conferred 100-fold higher levels of adhesion to umbilical vein endothelial and FaDu oral epithelial cells (Fu et al., 1998). *C. albicans* adhesin Eap1 has homology to cell wall proteins Hwp1 of *C. albicans*, as well as Flo11 and Aga1 of *S. cerevisiae* (Li and Palecek, 2003). Eap1 has been shown to enhance attachment of *C. albicans* to kidney epithelial cells, and confers this function upon *S. cerevisiae* when heterologously expressed. While only 1% of the *S. cerevisiae* cells carrying empty vector were able to adhere to kidney epithelial cells, over 35% of the Eap1-expressing cells were adherent, and this was similar to WT *C. albicans*. Expression of Eap1 also conferred a filamentous phenotype in *S. cerevisiae* (Li and Palecek, 2003). Alongside direct attachment to host cells, *C. albicans* is able to target ECM proteins, and one such protein that facilitates this is Tdh1. Tdh1 is expressed at the cell surface of *C. albicans* both *in vitro* and in samples of infected tissue collected from patients with systemic candidiasis (Gil-Navarro et al., 1997;Gil et al., 1999), and is capable of binding laminin and fibronectin (Villamon et al., 1999).

Other *C. albicans* adhesins are exclusively expressed on the surface of hyphae, such as Hwp1, Als3 and Ssa1 (Hoyer et al., 1998a;Staab et al., 1999;Sun et al., 2010). Hwp1 is necessary for *C. albicans* adhesion to buccal epithelial cells. A mutant strain was reduced in adhesion and was less able to initiate systemic infection in a mouse model of infection (Staab et al., 1999). Hwp1 is hypothesised to act as a target for host transglutaminases,

enabling formation of cross links between hyphae and host cells (Sundstrom et al., 2002). Severe systemic disease and subsequent killing was observed in immunodeficient mice infected orally with WT *C. albicans*, while no infection was established with a Hwp1 null mutant (Sundstrom et al., 2002). Deficiency of heat shock protein Ssa1 also led to reduced virulence in a mouse model of systemic and oral candidiasis, as well as inducing markedly less endothelial and oral epithelial cell damage *in vitro* (Sun et al., 2010). The *C. albicans* Δ*ssa1* mutant was less able to induce endocytosis by host cells, and this was attributed to an inability to bind N-cadherin and E-cadherin on endothelial and epithelial cells, respectively (Sun et al., 2010). Ssa1 has also been found to serve as a receptor for antimicrobial host protein histatin-5, and is necessary for fungal cell killing by defensins (Vylkova et al., 2006). This suggests that, although Ssa1 is important for *C. albicans* virulence, the host targets Ssa1 for clearance of the fungus.

Upon contact with oral epithelial cells *in vitro*, *C. albicans* has been shown to significantly upregulate expression of Als3 and Hwp1 (Wachtler et al., 2011), and Als3 has similarly been found to be frequently expressed *in vivo* by *C. albicans* found in vaginal fluid samples from patients with VVC (Cheng et al., 2005a). Loss of Als3 caused a reduction in adhesion to vascular endothelial cells of between 42% and 63%, depending on *C. albicans* strain, and around 60% in adhesion to buccal epithelial cells, signifying that Als3 is an important adhesin (Zhao et al., 2004b). To further support this, there was a significant reduction in numbers of *C. albicans* Δ*als3* able to colonise reconstituted oral epithelia, at an average of 0.1-0.2 *C. albicans* Δ*als3* cells attached per 250 µm tissue section, compared to 5.5 cells for WT, and this was observed alongside an ablation of epithelial cell damage in the mutant strains (Zhao et al., 2004b).

1.4.4.2 Toxicity

In addition to binding to mucosal epithelia, *C. albicans* is able to damage such tissues in a process that is associated with hyphal tissue penetration. Experiments using a range of *C. albicans* mutant strains found that only hyphae-producing strains initiated epithelial cell damage via phosphorylation of MKP1, c-Fos, neutrophil infiltration and cytokine release. However, one hyphae-producing strain deficient in *ECE1* was unable to induce damage, despite similar adherence and invasion levels to WT (Moyes et al., 2016). It was subsequently discovered that *ECE1* encodes a cytolytic pore-forming toxin, named

candidalysin. Candidalysin has been shown to be responsible for the proinflammatory response and pathology observed in vaginal thrush (Richardson et al., 2017). Reconstituted vaginal epithelial cells produced proinflammatory cytokines such as IL-1α, IL-1β and IL-8 in response to candidalysin, and there was dose-dependent release of LDH from A431 cells in response to candidalysin (Richardson et al., 2017). There was also a significant reduction in the number of neutrophils infiltrating the vaginal tissue of mice infected with *C. albicans* lacking Ece1, while a strain complemented with the locus encoding candidalysin restored neutrophil infiltration to WT levels (Richardson et al., 2017). Infiltration of neutrophils has not been correlated with clearance of fungal burden, but has instead been associated with a worsening of symptoms (Yano et al., 2014;Yano et al., 2012). Release of components of antifungal defence such as antimicrobial peptides and S100 alarmins was also dependent on candidalysin expression (Richardson et al., 2017). Similarly, production of IL-36 by oral epithelial cells was induced in response to candidalysin (Verma et al., 2018).

1.5 Interkingdom interactions

Biofilms are communities of microorganisms which attach to a surface and are surrounded by extracellular polymeric substance (EPS) (Harriott and Noverr, 2011). Polymicrobial biofilms are very common throughout the human body. Most microorganisms are likely to exist in biofilms, rather than as free, planktonic cells (Douglas, 2003;El-Azizi et al., 2004), and the National Institutes of Health in America estimates that 80% of infections are caused by microorganisms present in biofilms (NIH, 2002). This reflects the fact that biofilms increase the likelihood of microbial resistance to antimicrobial agents or components of the host immune system (Douglas, 2003;Lewis, 2001;Costerton et al., 1999) due, in part, to protection afforded by the EPS.

Polymicrobial biofilms involving *C. albicans* form at many sites within the human body (El-Azizi et al., 2004), including on catheters, dentures, or living tissue (Harriott and Noverr, 2011;Andes et al., 2004), and approximately 27-56% of hospital-acquired bloodstream infections caused by *C. albicans* involve other microorganisms (Harriott and Noverr, 2011). This reflects the capacity for *C. albicans* to mediate interspecies interactions with other yeasts or bacteria on both a physical (coaggregation/coadhesion) and chemical level, some examples of which are detailed below.

1.5.1 *C. albicans* interactions with *Staphylococcus aureus*

There is a well-documented synergistic interaction between *C. albicans* and *S. aureus*. *S. aureus* exhibits a strong tropism for *C. albicans* filaments, and the binding of hyphae allows *S. aureus* to "piggyback" into host cells, facilitating systemic disease (Schlecht et al., 2015b). This is the primary mechanism by which *S. aureus* can invade host cells (Schlecht et al., 2015b). Binding of *S. aureus* to Als3 on the surface of candidal hyphae is essential for this interaction to occur, as there was a significant decrease in *S. aureus-C. albicans* interactions when incubated with the Δ*als3* strain (Peters et al., 2012). In a mouse model of oral infection, mice incubated with only *C. albicans* exhibited symptoms of oral candidiasis but not invasive infection, while mice infected with only *S. aureus* showed no symptoms of disease. Mice infected with both *C. albicans* and *S. aureus* developed severe invasive disease and both microorganisms were isolated from the kidneys (which did not happen for either microorganism after single species infection) (Schlecht et al., 2015b). Furthermore, a mouse model of peritonitis showed that when *C. albicans* and *S. aureus* were both used to infect mice, infection was lethal in 40% of mice, compared to 0% for monomicrobial infection, and there was an increase in numbers of microbes present in the spleen and kidney (Peters and Noverr, 2013). This difference in mortality appeared to be driven by an enhanced inflammatory response to the dual-speces infection. There was a marked increase in production of proinflammatory cytokines and infiltration of neutrophils into host tissues in the polymicrobial versus the monospecies infected mice (Peters and Noverr, 2013). When mice were treated with a non-steroidal anti-inflammatory, there was a reduced production of proinflammatory cytokines and a reduction in neutrophil infiltrate and all mice survived, despite no inhibition in microbial growth *in vitro* (Peters and Noverr, 2013).

The biofilm matrix of *C. albicans* prevents infiltration of antibiotics into biofilms and this has been shown to promote tolerance of *S. aureus* to antibiotics. This was found to specifically be due to secretion of β-1,3-glucan. In dual-species biofilms, *S. aureus* was shown to become coated in *C. albicans* β-1,3-glucan, and this afforded protection against vancomycin. *S. aureus* could be sensitised to vancomycin by addition of caspofungin, an antifungal which inhibits synthesis of β-1,3-glucan (Kong et al., 2016).

1.5.2 *C. albicans* interactions with oral streptococci

C. albicans has been described to interact with a number of streptococcal species, particularly those that inhabit the oral cavity. This includes interactions with *Streptococcus gordonii*, *Streptococcus mutans*, and *Streptococcus oralis*. *S. gordonii* has been shown to secrete nutrients which are beneficial for *C. albicans* hyphae formation, promoting filamentation and biofilm formation (Bamford et al., 2009;Jenkinson et al., 1990). This effect was also significantly impaired when autoinducer-2 of *S. gordonii*, a quorum sensing molecule, was not expressed (Bamford et al., 2009). In addition, *C. albicans* hyphae formation is induced by environmental hydrogen peroxide (Nasution et al., 2008), which *S. gordonii* is capable of generating (Barnard and Stinson, 1996). Levels of hydrogen peroxide naturally produced by *S. gordonii* are typically lower than those found to be necessary for an effect on *C. albicans* (Bamford et al., 2009), but concentrations may be elevated if the two microbes are in close proximity. This appears to be a synergistic relationship, as *C. albicans* similarly benefits *S. gordonii* through production of an environment with lower levels of oxygen (Shirtliff et al., 2009). Both microbes also benefit from their ability to coadhere, which can facilitate their binding and retention within the oral cavity. This physical interaction is mediated, in large part, by *S. gordonii* cell surface protein SspB specifically recognising *C. albicans* hyphal adhesin Als3 (Silverman et al., 2010). Accumulation of salivary proteins such as proline-rich proteins on the surface of *S. gordonii* has also been shown to facilitate interaction with *C. albicans* (O'Sullivan et al., 2000).

Physical interactions that promote biofilm formation between *S. mutans* and *C. albicans* have been identified. In this instance, *S. mutans* glycosyltransferase GtfB attaches to *C. albicans* cell surface α-mannans, and this was shown to enable multispecies biofilm formation in a rat model of oral infection (Hwang et al., 2017). Interkingdom biofilms were less established when *S. mutans* was grown with *C. albicans* strains deficient in *N*- or *O*-mannan, or when WT *C. albicans* was grown with a *S. mutans* GtfB null mutant (Hwang et al., 2017). A study investigating the effects of *C. albicans* on the *S. mutans* transcriptome also found that there was significant upregulation of transcripts relating to carbohydrate metabolism (He et al., 2017). *S. mutans* is an important causative agent of dental caries, in which acid generation following bacterial metabolism of sugars leads to destruction of the mineralised tooth surface. Such an upregulation of *S. mutans*

transcripts relating to sugar catabolism by *C. albicans* may underpin the association of *C. albicans* and *S. mutans* in cases of severe early childhood caries (ECC) (He et al., 2017).

S. oralis is unable to form robust, monospecies biofilms within the oral cavity. However, when co-incubated with *C. albicans*, biofilm biomass of *S. oralis* was increased by 45-fold (Diaz et al., 2012). *S. oralis* in turn promoted oral mucosa invasion by *C. albicans* (Diaz et al., 2012), and facilitated *C. albicans* hyphae formation via upregulation of *EFG1* gene expression in polymicrobial biofilms *in vitro* and *in vivo* (Xu et al., 2017). This synergistic interaction between *C. albicans* and *S. oralis* facilitated degradation of E-cadherin from oral epithelial tight junctions, allowing systemic spread of *C. albicans* in a mouse model of infection (Xu et al., 2016).

Taken together, these studies serve to demonstrate that not only do *C. albicans*-streptococcal interactions promote adherence to host surfaces, they can facilitate establishment of disease.

1.5.3 *C. albicans* interactions with *Pseudomonas aeruginosa*

Not all polymicrobial interactions are synergistic, and a notable antagonistic relationship is that of *C. albicans* and *P. aeruginosa*. *P. aeruginosa* displays a tropism for *C. albicans* hyphae, which it binds to and kills (Hogan and Kolter, 2002). Binding to *C. albicans* filaments facilitated biofilm formation by *P. aeruginosa*, possibly via the bacteria harnessing nutrients from the fungus. This was supported by the observation that fungal cell death occurred only after commencement of biofilm formation (Hogan and Kolter, 2002). Pyocyanin, a virulence factor of *P. aeruginosa*, has been shown to inhibit transition of blastospores to hyphae in *C. albicans* via reduction of intracellular cAMP concentrations (Kerr et al., 1999). *P. aeruginosa* also out-competes *C. albicans* for nutrients. When in the presence of *C. albicans*, *P. aeruginosa* upregulates proteins which sequester iron, and this leads to a reduction in *C. albicans* metabolism (Purschke et al., 2012).

This relationship is not one sided, however. Farnesol production by *C. albicans* reduces production of pyocyanin by *P. aeruginosa* (Cugini et al., 2007) and can prevent *P. aeruginosa* swarming motility (McAlester et al., 2008). If *C. albicans* is present prior to *P.*

aeruginosa, the burden of *P. aeruginosa* in mouse lung tissue is significantly reduced, as is the presence of pathological lesions (Ader et al., 2011). This protection is mediated by activation and infiltration of NK cells, innate lymphoid cells, dendritic cells and macrophages into infected sites, and this was dependent on IL-22 secretion (Mear et al., 2014). A murine model of GI tract infection found that *C. albicans* inhibits biosynthesis of siderophores and cytotoxic virulence factors of *P. aeruginosa* via an unidentified secreted protein. This led to a significant reduction in *P. aeruginosa* virulence and an increase in survival to almost 100% (Lopez-Medina et al., 2015).

1.6 Hypothesis and objectives

Both *C. albicans* and GBS are common colonisers of the female GU tract. Given the precedent for *C. albicans* to exhibit synergistic interactions with Gram-positive bacteria, including streptococci, it was hypothesised that a similar relationship may occur between *C. albicans* and GBS. Such interactions could have potential to modulate the colonisation or pathogenic capabilities of both of these microbes. Nonetheless, there was only limited understanding of this relationship. The aims of this study were therefore to better characterise the molecular basis of interactions between *C. albicans* and GBS and the capacity for such interactions to influence microbial association with vaginal epithelial cells (VECs). This, in turn, could have implications for disease risk for both opportunistic pathogens. Specifically, the objectives of this study were:

i) To investigate whether GBS association with VECs is affected by *C. albicans*
ii) To investigate whether *C. albicans* association with VECs is affected by GBS
iii) To investigate the molecular basis for any interkingdom interactions
iv) To investigate the effects of any GBS-*C. albicans* interactions on the host

Chapter 2 Materials and Methods

2.1 Bacterial strains and culture conditions

The bacterial strains used in these studies are listed in Table 2-1. GBS was cultured in 10 mL THY broth (3.64% Todd-Hewitt powder [Oxoid], 0.5% Yeast Extract [Oxoid]) in sterile glass universals or maintained on THY agar plates at 37 °C, 5% CO_2. Media was supplemented with the following antibiotics as needed: 2 µg/mL chloramphenicol (Sigma), 5 µg/mL erythromycin (Sigma).

C. albicans was cultured in 10 mL YPD broth (1% Yeast Extract, 2% Mycological peptone [Oxoid], 2% D-glucose) in sterile conical flasks incubated at 37 °C, 220 rpm or maintained on Sabouraud dextrose agar (SAB) plates (Lab M) incubated aerobically at 37 °C.

L. lactis was cultured in 10 mL GM17 broth (3.725% M17 broth powder [Oxoid], 0.5% D-glucose) in sterile glass universals or maintained on GM17 agar plates at 30 °C in a sealed candle jar. Media were supplemented with 5 µg/mL erythromycin, and heterologous protein expression was induced by the addition of 0.1 µg/mL nisin.

S. cerevisiae was cultured in 10 mL Complete Supplement Media (CSM) without Uracil (0.077% CSM [Formedia], 0.67% Yeast Nitrogen Base, 2% D-glucose) in sterile conical flasks incubated at 30 °C, 220 rpm or maintained on CSM agar plates incubated aerobically at 30 °C.

Table 2-1 List of microbial strains used in experiments

Organism	Unique Reference Number	Strain / Relevant genotype	Reference/Source
C. albicans	UB2947	SC5314	Neil Gow (University of Aberdeen)
	UB1941	SC5314; Δ*als3*	(Zhao et al., 2004a)
	UB1940	SC5314; Δ*als3*+pUL.*als3*	(Zhao et al., 2004a)
S. agalactiae	UB1931	NEM316	Shaynoor Dramsi (Institut Pasteur)
	UB2416	18RS21	Shaynoor Dramsi (Institut Pasteur)
	UB2417	2603V/R	Shaynoor Dramsi (Institut Pasteur)
	UB2410	515	Victor Nizet, Univ California San Diego

	UB2873	515; Δ*bspC*	(Pidwill et al., 2018), see Appendix B: Published manuscript
	UB2874	515; Δ*bspC*+pDC.*bspC*	(Pidwill et al., 2018), see Appendix B: Published manuscript
	UB2414	COH1	Shaynoor Dramsi (Institut Pasteur)
	UB2866	COH1	Kelly Doran (San Diego State University)
	UB2919	COH1; Δ*bspC*	Kelly Doran (San Diego State University)
	UB2875	COH1; Δ*bspC*+pDC.*bspC*	Sara Rego (University of Bristol)
L. lactis	UB2635	NZ9800; pMSP	(Rego et al., 2016b)
	UB2658	NZ9800; pMSP.*bspA*	(Rego et al., 2016b)
	UB2659	NZ9800; pMSP.*bspC*	(Pidwill et al., 2018), see Appendix B: Published manuscript
S. cerevisiae	UB2161	BY4742; pBC542-*CWP1*	(Nobbs et al., 2010)
	UB2156	BY4742; pBC542-*ALS3sm*	(Nobbs et al., 2010)
E. coli	UB2464	BL21; pET46.*bspA*	Angela Nobbs (University of Bristol)
	UB2532	BL21; pET46.*bspC*	Angela Nobbs (University of Bristol)

2.2 Generation of GBS BspC mutants and *L. lactis* surrogate expression strains

Mutant strains were developed by Dr Sara Rego and Dr Angela Nobbs.

Using a previously described method (Maisey et al., 2007), Δ*bspC* mutants were produced in GBS strains 515 and COH1 by in-frame allelic replacement with a chloramphenicol resistance gene cassette by homologous recombination. To do this, flanking regions directly upstream and downstream of the *bspC* gene were amplified from genomic DNA using primer pairs *bspC*.F1/*bspC*.R1 and *bspC*.F2/*bspC*.R2, respectively (Table 2-2). Primers *cat*.F and *cat*.R were used to amplify the *cat* cassette from the chloramphenicol resistance plasmid pR326. Using stitch PCR, primers *bspC*.F1 and *bspC*.R2 were used to combine upstream and downstream *bspC* and *cat* amplicons. The resultant amplicon was cloned into vector pHY304 (53) via XbaI and BamHI sites and

propagated in E. coli Stellar cells (Clontech) prior to isolation and electroporation into GBS 515 or COH1 lacking the *bspC* gene.

L. lactis expressing BspA and BspC were generated as described previously (Rego et al., 2016b). Briefly, the *bspA* or *bspC* gene was amplified from GBS genomic DNA using primer sets pMSP.*bspC*.F and pMSP.*bspC*.R or pMSP.*bspA*.F and pMSP.*bspA*.R (Table 2-2). This was then cloned into pMSP7517, a nisin-inducible expression vector, via NcoI and XhoI sites to generate plasmids pMSP.*bspA* and pMSP.*bspC*. The vector was transformed directly into electrocompetent L. lactis NZ9800 as described previously (Rego et al., 2016b).

Transformants were confirmed by plasmid isolation and PCR, while expression of BspC in L. lactis was verified by dot immunoblotting.

Table 2-2 Primers used for mutagenesis

Primer name	Sequence*
bspC.F1	GCTCTAGAGCAATTAGCAGATGCACAG
bspC.R1	TAAAATCAAAGGAGAAAATATGAACTTTA
bspC.F2	GCTTTTATAATCAATATTCAGAAGCACTTG
bspC.R2	CGGGATCCGAGCCAAATTACCCCTCC
cat.F	AGAAAATATGAACTTTAATAAAATTGATTTAG
cat.R	TGAATATTGATTATAAAAGCCAGTCATTAGG
pMSP.*bspA*.F	CATGCCATGGAGGAGGAAACAATATGAATTCAC
pMSP.*bspA*.R	CCGCTCGAGGCAACCCGATTATGAGAGG
pMSP.*bspC*.F	CATGCCATGGAGGAGGAAATATGTATAAAAATCAAAAC
pMSP.*bspC*.R	CCGCTCGAGGCAGGTCCAGCTTCAAATC

*Restriction endonuclease sites are underlined.

2.3 Tissue culture

Experiments were conducted using VK2/E6E7 cells (ATCC CRL-2616), an immortalised human vaginal epithelial cell line that behaves similarly to the natural tissue (Steele and Fidel, 2002;Donnarumma et al., 2014;Sheen et al., 2011;Fichorova et al., 2011). Vaginal epithelial cells (VECs) were cultured in K-SFM (Keratinocyte Serum-Free Medium [Gibco®]) supplemented with 0.4 mM $CaCl_2$, 0.125 mg Bovine Pituitary Extract and 0.1 ng Epidermal Growth Factor. Cells were cultured in 75 cm^2 flasks (Corning®) at 37 °C, 5%

CO_2 and were passaged upon reaching 70-80% confluence, as per ATCC recommendations. Briefly, VECs were incubated with 3 mL TrypLE™ trypsin replacement enzyme (Fisher) for 15 min. TrypLE™ was neutralised with 7 mL Dulbecco's Modified Eagle's Medium (DMEM [Sigma]) supplemented with 10% Foetal Bovine Serum (FBS [Sigma]), and VECs were harvested. Cells were seeded into a fresh 75 cm^2 flask or 24-well plate, as appropriate.

2.4 Epithelial association assay

Microbial association assays were carried out similarly to those by (Sheen et al., 2011), with some modifications. VECs were seeded into a 24-well plate (Corning®) at $2x10^5$ cells/well, as determined by a cell counter, and incubated at 37 °C, 5% CO_2 until cells formed a confluent monolayer (24-48 h). An overnight broth culture of *C. albicans* was harvested (5000 *g*, 5 min), washed once in PBS (Lonza) and the pellet was suspended and adjusted to an OD_{600} 1.0 in K-SFM before 1:2 dilution into K-SFM to give approximately $5x10^5$ cells/mL. GBS or *L. lactis* cultures were similarly prepared and the adjusted suspension diluted 1:200 into K-SFM to give a suspension of approximately $5x10^5$ cells/mL. Wells containing VEC monolayers were washed once with PBS and, for monospecies assays, approximately $5x10^5$ bacteria or *C. albicans* were then added to each well (MOI 2.5). Bacterial suspensions were incubated at 37 °C, 5% CO_2 for 1 h, whilst *C. albicans* suspensions were incubated for 2 h. In dual-species assays, *C. albicans* suspensions were incubated for 1 h (with fresh K-SFM in control wells), then removed prior to addition of either GBS or *L. lactis* and incubated for a further 1 h. For all assays, wells were then washed three times with PBS. The plate was incubated at 37 °C, 5% CO_2 for 15 min following addition of 200 µL of TrypLE™ to disperse the cells, followed by two 20 min incubations with ice cold water (500 µL) to lyse the epithelial cells. Lysates were transferred to 1.5 mL microcentrifuge tubes, vortexed for 10 s to mix and serially diluted with appropriate broth. Numbers of associated GBS, *L. lactis* or *C. albicans* were then determined by viable count. Lysates from dual-species assays were pipetted onto either THY agar plates (GBS) or GM17 agar plates (*L. lactis*) supplemented with 50 µg/mL nystatin (Sigma) to inhibit *C. albicans* growth. For experiments which required the colony forming units per mL (CFU/mL) of *C. albicans* to be determined, lysates were plated onto SAB agar plates supplemented with 5 µg/mL erythromycin to inhibit

bacterial growth. All plates were incubated overnight before colonies were counted and numbers of associated bacteria (CFU/mL) determined.

2.5 Invasion assay

Invasion assays were carried out to determine the number of GBS or *L. lactis* cells that internalised into VEC monolayers during an epithelial association assay. Cells were inoculated with a ten times higher inoculum for GBS assays (MOI 25), or 100 times higher for *L. lactis* (MOI 200), to allow reliable detection of numbers of internalised bacteria. The invasion assay was performed as described above (section 2.4) with the exception that, following incubation of VEC monolayers with microbes, monolayers were incubated with 0.5 mL K-SFM supplemented with 200 µg/mL gentamicin (Sigma) and 10 µg/mL penicillin G (Sigma)]. These antibiotic concentrations were similar to those described in the literature as sufficient to kill GBS (Stoner et al., 2015;Sheen et al., 2011), and this was verified prior to use in the assays. Plates were then incubated at 37 °C, 5% CO_2 for 2 h, before monolayers were washed with PBS and lysed as described above. Any bacteria that remained viable were assumed to have invaded the VECs and thus been protected from the antibiotics. To investigate the effects of longer incubation times on invasion, after 1 h incubation with GBS, media were removed and 1 mL of fresh K-SFM was added to each well. VECs were then incubated for a further 4 h before addition of antibiotic-supplemented K-SFM, as described above.

2.6 Gram stain of VEC association assay

In a variation of the association assay, VECs were grown on sterile 19 mm round glass coverslips, seeded at $2x10^5$ cells/well, and grown at 37°C, 5% CO_2 until confluent (48-72 h). The assay was carried out as described in section 2.4 up to the final PBS washes, after which coverslips were air-dried and heat-fixed. The coverslips were then Gram stained and mounted with DPEX (VWR). Gram stained samples were visualised by light microscopy on a table-top Leica DMLB microscope.

2.7 Confocal microscopy

In a variation of the association assay, VECs were grown on sterile 19 mm round glass coverslips, seeded at $2x10^5$ cells/well, and grown at 37°C, 5% CO_2 until confluent (48-72 h). The assay was carried out as described in section 2.4 up to the final PBS washes, after which calcofluor white (0.00001% in dH_2O) was added to coverslips to stain chitin in the *C. albicans* cell wall. Coverslips were fixed in 2% paraformaldehyde (PFA) for a minimum of 45 min. Epithelial cells were permeabilised with 0.3% Triton X-100 for 10 min before blocking in 2% BSA for 45 min. Coverslips were then incubated with mouse anti-GBS antibody (0.5 µg/mL, Santa Cruz Biotechnology), before incubation with goat anti-mouse Alexafluor-488 (10 µg/mL, Fisher). Epithelial cells were labelled with phalloidin conjugated to TRITC (Sigma), which stains F-actin. Vectashield (Vector labs) was used to mount coverslips onto glass slides and nail varnish was used to fix coverslips in place. Coverslips were imaged on a Leica SP5-AOBS confocal scanning laser microscope (CSLM) attached to a Leica DM I6000 inverted epifluorescence microscope. Images were processed using Volocity® software and Imaris® v7.5 software (Bitplane AG) was used to calculate biovolumes (μm^3).

2.8 Transwell studies

In a variation of an association assay (section 2.4), *C. albicans* was prepared as described above, and incubated with VECs for 1 h at 37 °C, 5% CO_2. GBS was prepared as described (section 2.4) and was added to the top compartment of transwell inserts with high density 0.4 µm pores (Sarstedt) suspended above wells containing *C. albicans* and VECs. This was incubated for 1 h at 37 °C, 5% CO_2. Cells were washed, lysed and lysate serially diluted as described for an association assay onto SAB agar for determination of *C. albicans* CFU/mL.

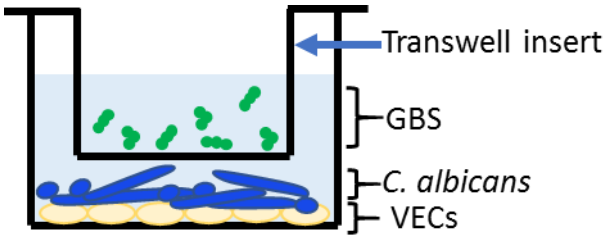

Figure 2-1 Schematic of a transwell.

Schematic showing a tissue culture well with a transwell insert. VECs (yellow) are seeded into a 24 well plate and grown to confluence before incubation with *C. albicans*(blue). A transwell insert is added to the well, and GBS (green) is added to the top compartment.

2.9 Conditioned media studies

In a variation of an association assay (section 2.4), *C. albicans* or GBS were prepared as described above, and incubated separately on VECs for 1 h at 37 °C, 5% CO_2, or at the same concentration planktonically at 37 °C, 220 rpm. Media was pooled and filter sterilised.

C. albicans or GBS were harvested from an overnight broth culture, washed once in 5 mL PBS and adjusted to OD_{600} 1.0 in K-SFM. This suspension was diluted 1:2 (*C. albicans*) or 1:200 (GBS) into either blank K-SFM, or sterilised media from either planktonic or cell-grown *C. albicans* or GBS, as appropriate, and incubated with VECs at 37 °C, 5% CO_2 for 1 h. Cells were washed, lysed and the lysate serially diluted onto appropriate agar to determine viable counts, as for an association assay.

2.10 AgI/II inhibition assay

In a variation of an association assay, VECs were grown to confluence in 24 well plates. GBS was harvested from an overnight broth culture, washed in PBS (5 mL) and incubated for 30 min with 10 µg/mL anti-BspA or anti-BspC antibody (Eurogentec), as appropriate, or 10 µg/mL rabbit preimmune sera (Eurogentec) as negative control. GBS was harvested and adjusted to OD_{600} 1.0, before 1 mL of adjusted GBS suspension (MOI 2.5) was added to VECs and incubated for 1 h at 37 °C, 5% CO_2. Cells were lysed, and lysate was serially diluted onto agar to determine viable counts.

2.11 Visualisation of dual-species planktonic interactions

An overnight *C. albicans* broth culture was harvested at 5000 *g* for 5 min and washed once in YNBPT (0.67% Yeast Nitrogen Base [Appleton Woods], 20 mM Na_2HPO_4 adjusted to pH 7 with KH_2PO_4, 0.1% tryptone). The pellet was suspended and adjusted to OD_{600}

1.0 in YNBPT. The adjusted suspension was diluted 1:10 into YNBPTG (YNBPT supplemented with 0.4% glucose), 2 ml was transferred to a sterile glass bijou, and incubated at 37 °C, 220 rpm for 2 h before addition of bacteria.

An overnight GBS or *L. lactis* broth culture was harvested at 5000 *g* for 7 min, washed once in YNBPT, and the pellet was suspended in 1.5 mM fluorescein isothiocyanate (FITC) dissolved in carbonate buffer (0.53% Na_2CO_3, 0.59% NaCl), then incubated for 30 min at 50 rev/min to fluorescently stain the bacterial cells. Labelled bacteria were harvested, washed three times in carbonate buffer and the pellet suspended and adjusted to OD_{600} 0.5 in YNBPTG. GBS or *L. lactis* (1 mL) was added to the *C. albicans* and incubated for a further 1 h at 37 °C, 220 rpm. Calcofluor white was added to stain *C. albicans*, before visualisation of a 10-15 µL aliquot of the suspension by fluorescence microscopy using a table-top Leica DMLB microscope. For quantification assays, approximately 40 hyphae were randomly selected from each experimental group and imaged. Interactions were scored into one of four groups: 0 interacting bacteria per hyphae, 1-5 bacteria, 6-20 bacteria and more than 20 bacteria per hyphae. This was similar to semi-quantitation described by (Silverman et al., 2010).

2.12 *S. cerevisiae-L. lactis* interactions in suspension

In a variation of the above assay (section 2.11), *S. cerevisiae* was harvested from an overnight broth culture at 5000 *g* for 5 min and washed once in YNBPT (5 mL). Cells were then labelled in 1.5 mM FITC at 50 rev/min for 30 min to fluorescently stain the yeast cells. Stained *S. cerevisiae* was then harvested and washed in carbonate buffer three times. The pellet was suspended and adjusted to OD_{600} 1.0 in YNBPTG, diluted 1:5 into YNBPTG (final volume 2 mL), and incubated at 30 °C, 220 rpm for 3 h. *L. lactis* was harvested from an overnight broth culture at 5000 *g* for 7 min, washed once in YNBPT (5 mL) and stained with TRITC (0.1 mg/mL in carbonate buffer) for 30 min with gentle agitation. Labelled *L. lactis* was harvested, washed in carbonate buffer three times and adjusted to OD_{600} 0.5 in YNBPTG. 1 mL of this suspension was added to *S. cerevisiae* and microbes were incubated for a further 1 h at 30 °C, 220 rpm. 10 µL samples were visualised by fluorescence microscopy using a table-top Leica DMLB microscope.

2.13 VEC viability testing

Supernatants from association assays (section 2.4) were collected and investigated for the presence of lactate dehydrogenase (LDH) using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), according to manufacturer's specifications. Briefly, 50 µL of assay supernatant was mixed with 50 µL of reagent in a 96 well plate and incubated at room temperature for 30 min in the dark. The reaction was halted by the addition of 50 µL of Stop Solution, and the absorbance was measured at 490 nm by a fluorescence plate reader (iMark™ microplate absorbance reader, BioRad). To establish the maximum LDH release, lysis solution (Promega) was added to control cells. The percentage cell cytotoxicity was calculated by dividing experimental LDH release (OD_{490}) by the maximum LDH release (OD_{490}) and multiplying by 100.

To further demonstrate the viability of the VEC monolayer, brightfield images were taken with a table-top Olympus CKX41 inverted microscope at each stage of microbial association assays.

2.14 Microbial growth in dual-species broth cultures

To test the effect of co-incubation on microbial growth, microbes were incubated together in suspension at 37 °C, 220 rpm in K-SFM. *C. albicans* was harvested from an overnight broth culture and washed once in PBS before adjustment to an OD_{600} of 1.0 in K-SFM. The adjusted suspension was diluted 1:10 into K-SFM (2 mL final volume) and incubated in sterile glass bijious at 37 °C, 220 rpm for 2 h. GBS was harvested from an overnight broth culture, washed once in 5 mL PBS and adjusted to OD_{600} 0.5 in K-SFM. 1 mL of this suspension was added to the *C. albicans* bijious (or to 2 mL K-SFM for controls). Suspensions were incubated for a further 1 h at 37 °C, 220 rpm. Bijious were vortexed for 10-15 s before serial dilution into THY broth. CFU/mL were determined by plating onto appropriate agar.

2.15 Immunofluorescence staining of GBS

An overnight broth culture of GBS was harvested, washed once in PBS (5 mL), and adjusted to OD_{600} 1.0 in PBS. This suspension was harvested and resuspended in 4% w/v PFA(4 mL), before incubation at RT under gentle agitation for 30 min. The suspension was harvested and washed twice in PBS (1 mL) before incubation in 10% rabbit serum (1 mL) for 30 min at RT under gentle agitation. Cells were then washed once in PBS (1 mL) before incubation with primary antibody (either anti-BspA or anti-BspC, as appropriate; 1:200 dilution in PBS) for 1 h at RT under gentle agitation. Cells were washed twice more in PBS (1 mL), followed by incubation with anti-rabbit Alexafluor-555 secondary antibody (1:200 dilution in PBS) for 1 h at RT under gentle agitation. Cells were washed a final two times in PBS (1 mL), resuspended in 200 µL PBS, and 10 µL visualised by fluorescence microscopy using a table-top Leica DMLB microscope.

For experiments investigating the effects of temperature on AgI/II family protein expression, overnight broth cultures were set up at either 30 °C in a candle jar, 34°C or 37°C, 5% CO_2, while for experiments investigating the effects of growth phase, bacteria were harvested from overnight broth cultures grown at 37°C, 5% CO_2, washed once in PBS (5 mL), and adjusted to OD_{600} 1.0 in THY broth (5 mL) before dilution 1:10 into pre-warmed THY broth (15 mL). The optical density was tested every 30-60 min as appropriate until reaching OD_{600} 0.2-0.3 (early exponential) or OD_{600} 0.8-0.9 (late exponential). Cells were harvested and adjusted to OD_{600} 1.0 in PBS prior to fixation and labelling, as described above.

2.16 Dot blot

Aliquots (2 µL) of GBS overnight broth culture, alongside aliquots of five successive 1:2 dilutions, were transferred onto a nitrocellulose membrane and air dried. The membrane was blocked in Tris-buffered saline (TBS; 10 mM Tris, 0.15 M NaCl, pH 8) with 10% milk for 1 h. The membrane was washed once in TBST (TBS with 0.1% Tween-20) before probing with rabbit anti-BspC antibody diluted 1:100 in TBST with 1% milk for 1 h. After 3 washes in TBST, the membrane was probed with swine anti-rabbit HRP diluted 1:1000 in TBST with 1% milk for 1 h. The membrane was washed a further two times in TBST before a final wash in TBS. Enhanced chemiluminescence solution (GE Healthcare)

was transferred onto the blots and incubated for 1 min. Blots were exposed to X-ray film.

2.17 Real-time RT-PCR

VECs were grown to confluence in 75 cm^2 tissue culture flasks (Corning®) before incubation with *C. albicans* (10 mL, MOI 2.5) at 37 °C, 5% CO_2 for 4 h, or with GBS strain NEM316 or COH1 (10 mL, MOI 5) for 3 h. Alternatively, VECs were incubated with *C. albicans* for 1 h followed by GBS for a further 3 h. Flasks were washed three times with PBS to remove non-adherent microbes before TrypLE™ trypsin replacement enzyme (4 mL) was added and flasks were incubated at 37 °C, 5 % CO_2 for 15 min to suspend monolayers. DMEM with 10% FBS (4 mL) was added to neutralise the trypsin, and remaining adherent cells were scraped into solution using a cell scraper (Fisher). Cells were harvested at 800 *g* for 10 min, and RNA was extracted using the Qiagen RNeasy mini kit (Qiagen), as per manufacturer's instructions. Purified RNA was digested with RQ1 DNase (Promega), followed by RNA clean-up using the RNeasy mini kit, as per manufacturer's instructions. The concentration of RNA was measured at an absorbance of 260 nm using a Nanodrop spectrophotometer. RNA was converted to cDNA according to manufacturer's instructions using iScript Reverse Transcriptase (Biorad), which contains oligo (dT) and random primers, and incubated at 25 °C for 5 min, followed by 42 °C for 30 min, then 85 °C for 5 min, before being held at 4 °C. All cDNA samples were tested alongside equivalent NRT (no reverse transcriptase) control samples with GAPDH primers (Table 2-3) by end-point PCR to confirm successful cDNA synthesis and verify lack of DNA contamination in RNA preparations.

Once generated, cDNA was used for real-time qPCR using iSYBR green (Biorad), according to manufacturer's instructions, and seven primer sets (Table 2-3): IL-1α, IL-1β, IL-8, IL-17, IL-23, IL-36γ and GAPDH, the latter serving as endogenous control. A Biorad CFX Connect real time PCR machine was used with the following programme: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 seconds, 58 °C for 30 seconds and 72 °C for 30 seconds, and finishing with a melt curve analysis increasing by 0.5 °C from 58 °C to 95 °C. Data were analysed using the $\Delta\Delta C_t$ method. C_t value was defined as the change in the cycle number the data exceeded the significance threshold between a test sample for the endogenous control and the C_t value for the cytokine of interest, and this C_t

difference was taken away from the equivalent value for the control sample (in this case VEC only) with the resulting figure interposed to the power of 2 to generate the expression ratio.

Table 2-3 Primers used for RT-PCR

Name	Sequence (5'→3')	Target
GAPDH_F	GGAAGGACTCATGACCACAG	RT-PCR primer: GAPDH
GAPDH_R	TTGGCAGGTTTTTCTAGACG	RT-PCR primer: GAPDH
IL-1α_F	CCCAAAACCATCACAGGTAG	RT-PCR primer: IL-1α
IL-1α_R	GCACACCCAGTAGTCTTGCT	RT-PCR primer: IL-1α
IL-1β_F	AATCTCCGACCACCACTACA	RT-PCR primer: IL-1β
IL-1β_R	GAAAGAAGGTGCTCAGGTCA	RT-PCR primer: IL-1β
IL-8_F	GTTTTGCCAAGGAGTGCTAA	RT-PCR primer: IL-8
IL-8_R	CCAGACAGAGCTCTCTTCCA	RT-PCR primer: IL-8
IL-17_F	CTACAACCGATCCACCTCAC	RT-PCR primer: IL-17
IL-17_R	CCACGGACACCAGTATCTTC	RT-PCR primer: IL-17
IL-23_F	CAGTTCTGCTTGCAAAGGAT	RT-PCR primer: IL-23
IL-23_R	ATCTGCTGAGTCTCCCAGTG	RT-PCR primer: IL-23
IL-36γ_F	TGAGAAGGTTGGAGAACAGC	RT-PCR primer: IL-36γ
IL-36γ_R	GGTGGAGGTCCTACCAGTCT	RT-PCR primer: IL-36γ

2.18 Proteomics

VECs were grown to confluence in 75 cm^2 tissue culture flasks (Corning®) before incubation with *C. albicans* (10 mL, MOI 2.5) at 37 °C, 5% CO_2 for 4 h, or with GBS strain NEM316 (10 mL, MOI 10) for 3 h. Alternatively, VECs were incubated with *C. albicans* for 1 h followed by addition of GBS and incubation for a further 3 h. Monolayers were washed three times with PBS (5 mL) before 2 mL of 8 M urea was added. Cells were scraped into suspension, before centrifugation at 17000 *g* for 10 min, 4 °C. Supernatants were recovered and the protein concentration determined using a Bradford assay, before being snap frozen in liquid nitrogen and stored at -70 °C. Samples were then transported to the biochemistry proteomics facility, where proteins were precipitated using acetone before total protein was digested with trypsin.

Peptides were separated and identified by tandem mass-tagging (using Fisher 10-plex reagents) followed by mass spectrometry. Spectra were generated for each peptide which were used to identify the theoretical amino acid number, isoelectric point and molecular weight. This data was inputted into the FASTA database in order to identify the peptides. Peptide data were excluded if detected in only one of the two replicates

per sample, or if only one of the replicates exceeded the threshold value which was considered significant (i.e. either more than 2-fold increase or decrease in protein expression relative to VEC only control). Peptides which were significantly altered in expression in relation to the VEC control were then analysed.

2.19 Neutrophil isolation

Blood was collected from anonymous, healthy volunteers, who provided written informed consent. The University of Birmingham School of Biosciences Institutional Review Board approved the protocol for blood collection and isolation of neutrophils from healthy volunteers. 1.098 density Percoll (GE Healthcare) was layered under 1.079 density Percoll to create a sucrose gradient. Blood was layered on top of this, and separated by centrifugation at 150 *g* for 8 min, followed by 1200 *g* for 10 min. The neutrophil layer was collected and transferred to a fresh tube. Lysis buffer (0.83% ammonium chloride) was added, and cells were incubated at RT for 2 min to allow lysis of any contaminating red blood cells. Cells were harvested at 350 *g* for 6 min, and the pellet was suspended in 1 mL K-SFM. Neutrophils were counted using a haemocytometer, and cells were diluted into K-SFM as necessary.

2.20 Neutrophil chemotaxis

Neutrophils were tested for chemotaxis stimuli in response to supernatant harvested from VEC monolayers grown in 24-well plates that had been exposed to GBS strains NEM316, 515 or COH1 (MOI 2.5) for 5 h, or *C. albicans* (MOI 2.5) for 6 h, or *C. albicans* for 1 h followed by GBS for 5 h. Supernatant from each well was clarified by centrifugation (17000 *g*, 7 min), and 800 µL transferred to a sterile microcentrifuge tube for storage at -20 °C prior to use.

Neutrophils were stained with 250 nM Syto13 (Fisher) and incubated in the dark at RT for 20 min. Neutrophils were then harvested at 300 *g* for 4 min and suspended in 1 mL K-SFM with 0.01% BSA. Chamber slides (Ibidi) were loaded with neutrophils in the central viewing chamber, as per manufacturer's instructions. Neutrophils were

incubated at RT for 10 min to allow initial adherence before exposure to stimuli. For all chambers used, K-SFM was added to the left hand chamber, while 1:2 diluted test sample was added to the right hand chamber. 200 nM FMLP (Sigma) was used as a positive control alongside VEC supernatants. A microscope was set up with the viewing area of each of the chamber slides in a tile scan. Images were taken of each of the viewing areas every 2 min for 1 h (i.e. 31 time points per sample) using a Zeiss microscope. Stacks of images were analysed in Fiji imaging software (ImageJ), using the 'Trackmate' plugin to generate positional data about neutrophils in a stack of images. Neutrophils which could not be tracked for all 31 time points were excluded. These data were then subject to further analysis with the Ibidi Chemotaxis and Migration Tool software (Ibidi).

2.21 Purification of recombinant Bsp protein and antibody generation

E. coli strains expressing BspA and BspC protein (UB2464 and UB2532, respectively, Table 2-1) were inoculated into LB broth (100 mL) with 50 µg/mL carbenicillin and grown overnight at 37 °C, 180 rpm. Bacterial suspensions were then diluted 1:10 into LB broth with 50 µg/mL carbenicillin and grown to OD_{600} 0.6. IPTG was added to a final concentration of 1 mM to induce protein expression, and suspensions were grown at 18 °C, 180 rpm overnight. Bacteria were harvested at 5000 *g* for 15 min at 4 °C. Pellets were suspended in gel filtration buffer (20 mM Tris, 150 mM NaCl, pH 7.5), cells harvested at 4500 *g*, 15 min, 4°C, and pellets stored at -80 °C. Frozen pellets were suspended in 45 mL loading buffer (50 mM Tris, 150 mM NaCl, 20 mM imidazole, pH 7.5) with protease inhibitor cocktail (PIC; Sigma) and homogenised, before samples were passed through a cell disrupter. The resulting mixture was then centrifuged at 18000 *g* for 25 min at 4 °C. The supernatant was collected and loaded onto a nickel column (Sigma). Samples were processed by nickel affinity chromatography using an AKTA machine. Undesired proteins were washed off the column with loading buffer, while BspA or BspC was eluted with elution buffer (50 mM Tris, 150 mM NaCl, 1 M imidazole, pH 7.5). 10 µl aliquots of fractions that produced protein peaks were denatured at 95 °C for 5 min before being run on a pre-cast Tris-glycine SDS PAGE gel (NuSep) at 200 V for 40 min. Gels were stained with Coomassie Blue, and fractions with protein at ~100 kDa were combined and concentrated using a 50,000 molecular weight concentrator at 4500 *g* until the total

volume was <5 mL. Aggregated protein was harvested at 14000 *g* for 10 min, 4 °C, and the supernatant was loaded onto an AKTA machine for separation by size exclusion chromatography. 10 µl aliquots of fractions that produced protein peaks were analysed by SDS PAGE, as above. Fractions containing purified Bsp protein were pooled, dialysed into dH_2O with 0.1% PIC and 0.2% PMSF, concentrated as above, and stored at -80 °C.

Pre-immune sera was collected from potential rabbits and screened against BspA and BspC purified proteins to test for reactivity. Rabbits with no antigenicity to BspA/C were selected for antibody generation. To do this, Eurogentec immunised rabbits with purified protein four times, once on day one of the immunisation schedule, then days 7, 10 and 18. The final bleed was collected 28 days after initial immunisation and antibodies were purified from this.

2.22 Statistical analysis

Data were analysed by either Student's unpaired *t*-test (with Bonferroni correction for multiple groups) or by one-way ANOVA with Tukey post-test in SPSS Statistics (version 24; IBM), as indicated. All assays were independently repeated in triplicate with three experimental replicates unless otherwise stated.

Chapter 3 Interactions between GBS and *C. albicans*

3.1 Introduction

As detailed in Chapter 1, *C. albicans* is commonly found as a member of polymicrobial communities within the human host. Moreover, these polymicrobial interactions have often been shown to modulate the colonisation and pathogenic capabilities of the microorganisms involved. Such precedent meant that a similar dynamic may occur between *C. albicans* and GBS, which are both opportunistic pathogens of the female genital tract. An association between these two microorganisms was also supported by a number of clinical observations. Bayo et al. (2002) found that 54.5% of GBS isolated from the vaginal tract of pregnant women co-isolated with *C. albicans*, although relatively few (43) women were colonised with GBS (Bayo et al., 2002). In a larger study, 1284 vaginal swabs (32% of those collected) were identified as positive for GBS and of these, 457 (38%) were found to be colonised with *C. albicans* (Meyn et al., 2009). Consequently, *C. albicans* was described as a risk factor for GBS vaginal colonisation. This was also supported by earlier studies. Monif and Carson (1998) found that GBS was isolated from vaginal swabs more frequently with *C. albicans* than without (27.3% rather than 16%) (Monif and Carson, 1998). Likewise, risk of GBS vaginal colonisation in pregnancy was shown to be greater when *Candida* was co-isolated, with 35.8% of *C. albicans* positive swabs also testing positive for GBS (Regan et al., 1991). All of these studies focused on women in the US/Europe, but a comparable trend was also reported by a study of vaginal GBS carriage in Kenya and South Africa, where it was found that *C. albicans* colonisation was an independent risk factor for GBS vaginal colonisation, with 25% (17 of 67) of GBS-positive vaginal swabs also testing positive for *C. albicans* (Cools et al., 2016).

A correlation between occurrence of *C. albicans* and GBS had also been reported for studies from a candidal perspective. A study investigating vaginal colonisation by yeast (98% of which was identified as *C. albicans*), found that vaginal GBS co-colonisation was significantly associated with yeast colonisation (Beigi et al., 2004). Likewise, a study of *C. albicans* vaginal colonisation amongst pregnant women found that 36.8% (i.e. 419 of 1139 women) of *C. albicans*-positive vaginal swabs were also positive for GBS, compared to 19.5% in uncolonised women (Cotch et al., 1998).

The reported co-occurrence of GBS and *C. albicans* within the GU tract provided evidence that these two microorganisms had the potential to interact and modulate colonisation of the vaginal mucosa and thus subsequent disease risk. These studies therefore aimed to explore the relationship between GBS and *C. albicans*, and to determine what effect (if any) this had on colonisation of VECs.

3.2 Results

3.2.1 Planktonic interactions between GBS and *C. albicans*

A common mechanism by which two microorganisms may interact is via coaggregation under planktonic conditions. The first step of this project was therefore to establish whether GBS and *C. albicans* were capable of coaggregating. To enable this, 5 strains of GBS were investigated: NEM316, 18RS21, 2603V/R, 515 and COH1. These 5 strains were selected to cover the most common GBS capsular serotypes associated with disease (Table 3-1). This would then potentially enable any capsular polysaccharide (CPS) specific effects on coaggregation capabilities to be identified. As several bacterial species, including GBS, had been reported to exhibit a tropism for *C. albicans* hyphae rather than blastospores (Rego et al., 2016b;Peters et al., 2012;Silverman et al., 2010), *C. albicans* was induced to form hyphae by pre-incubation in growth medium supplemented with glucose. Each GBS strain was then incubated with *C. albicans* in suspension for 1 h. Microbes were fluorescently labelled with FITC (GBS, green) or Calcofluor White (*C. albicans*, blue), before visualisation by fluorescence microscopy (Figure 3-1A). Semi-quantitative analysis was also performed, for which images were taken of approximately 40 randomly-selected hyphae per replicate, and each hypha was allocated a score according to the number of GBS bound to it (Figure 3-1B).

Table 3-1 Overview of selected GBS strains used in these studies

GBS strain	CPS	CC	Pili
NEM316	III	23	PI-1 and PI-2a
18RS21	II	19	PI-1 and PI-2a
2603V/R	V	19	PI-1 and PI-2a
515	Ia	23	PI-2a
COH1	III	17	PI-1 and PI-2b

From these data it was apparent that GBS interactions with *C. albicans* were strain-dependent. GBS strains NEM316 and 515 exhibited the highest levels of coaggregation with *C. albicans*, with 51% and 43% of hyphae, respectively, observed with >20 interacting bacteria, while this was <3% for 18RS21, 2603V/R and COH1 (Figure 3-1). For GBS strains 18RS21, 2603V/R and COH1, the majority (86%, 80% and 84%, respectively) of *C. albicans* hyphae randomly selected for semi-quantitation had only 0 or 1-5 interacting bacteria (Figure 3-1). Despite these variations in coaggregation capacity, all of the GBS strains were observed to show a tropism for *C. albicans* hyphae rather than blastospores. This supported data previously described by (Rego et al., 2016b) for GBS strain NEM316.

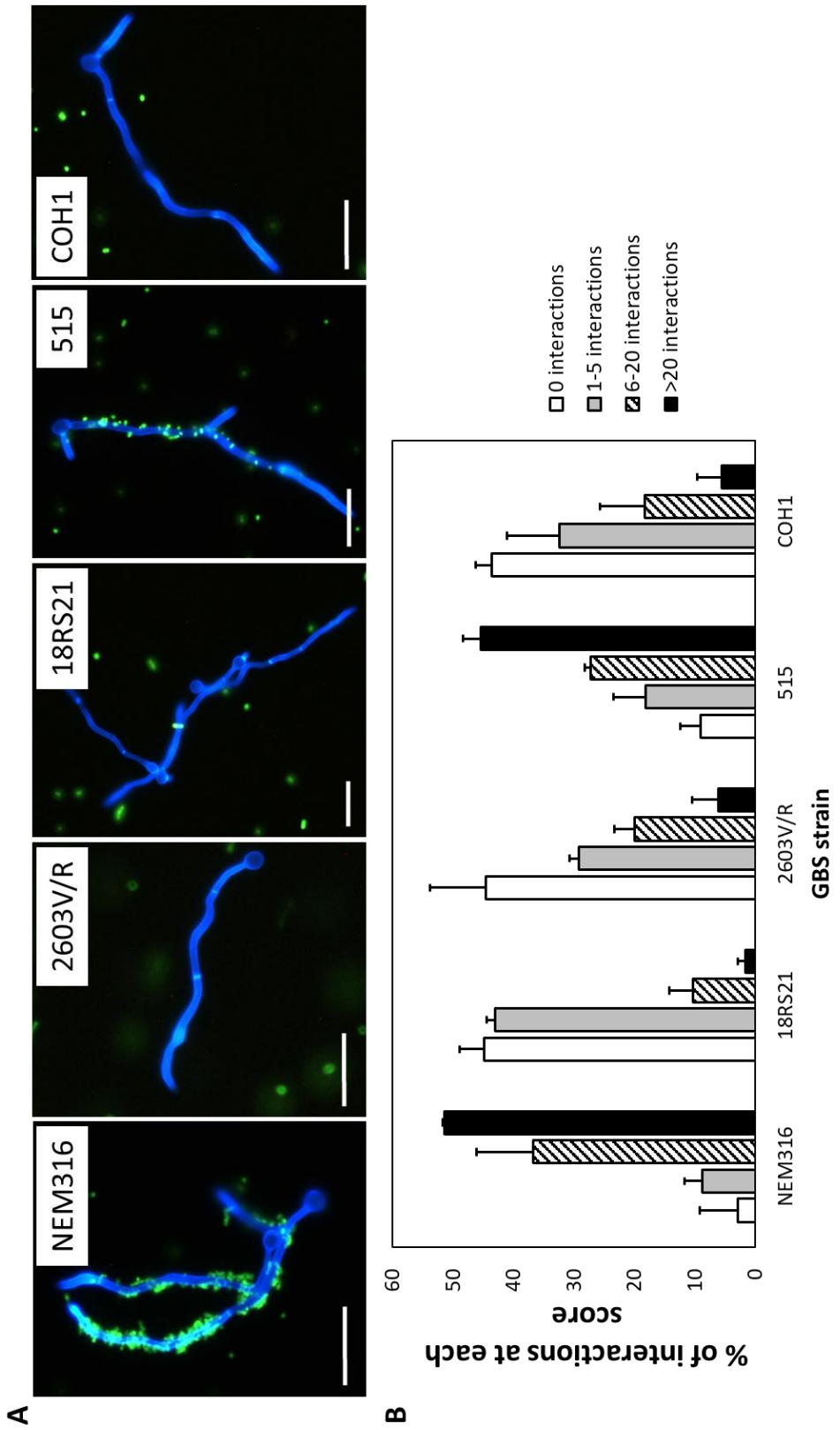

Figure 3-1 Planktonic suspension interactions between GBS and *C. albicans*.

A) Representative fluorescence micrographs of planktonic interactions between *C. albicans* and GBS. *C. albicans* was grown in YNBPTG for 2 h at 37 °C, 220 rpm before addition of GBS strain NEM316, 2603V/R, 18RS21, 515 or COH1 (as indicated) and incubation for a further 1 h. GBS was labelled with FITC (green), while *C. albicans* was labelled with Calcofluor White (blue). Scale bars, 20 µm. B) Semi-quantitation of numbers of *C. albicans* hyphae with 0 interacting GBS, 1-5, 6-20 or >20 interacting GBS; data are presented as mean ± SD; n=2.

3.2.2 Association of GBS with VK2/E6E7 cells

As this project was ultimately focused on the interactions of GBS and *C. albicans* with vaginal epithelium, it was also important to determine the capacity for each GBS strain to associate with vaginal epithelial cells. For these studies, vaginal epithelial cell (VEC) line VK2/E6E7 was used. This cell line has been generated from vaginal mucosa obtained from a premenopausal woman during routine surgery, which was immortalised with the E6 and E7 proteins from human papillomavirus (Fichorova et al., 1997). VECs were incubated with GBS for 2 h before VECs were disassociated and lysed, and numbers of associated GBS determined by viable count following plating of the lysates onto agar. There were significant differences in association between the GBS strains, as indicated in Figure 3-2, with overall numbers of recovered GBS ranging from $1.3x10^6$ to $3.2x10^6$. Strains 18RS21 and 2603V/R exhibited the highest levels of association, followed by COH1 and NEM316. Strain 515 exhibited the lowest level of association of the GBS strains.

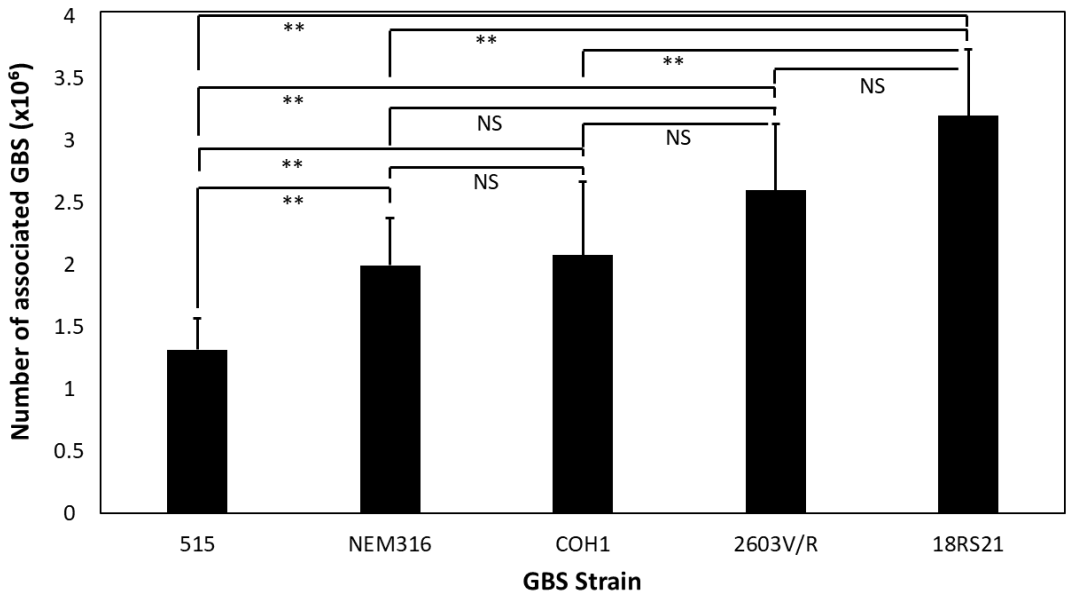

Figure 3-2 Association of GBS strains with VECs.

VEC monolayers were incubated with GBS suspensions (MOI 2.5) for 2 h. Monolayers were then lysed and numbers of associated GBS enumerated by viable count. Data are presented as mean ± SD, ** indicates $P<0.005$, as determined by one-way ANOVA with a Tukey post-hoc test; n = 4.

GBS interactions with VECs were also visualised by Gram stain, in which VEC monolayers were stained pink with safranin, while GBS bacteria stained purple with crystal violet (Figure 3-3). Corresponding with the CFU/mL data displayed in Figure 3-2, GBS strains 18RS21 and 2603V/R were observed in greater numbers than NEM316, 515 and COH1. It was also noted that strain 2603V/R formed longer chains of bacteria than the other strains. Strain 515 was observed mainly interacting with 'giant' cells, which possibly represented cells that had failed to go through successful cytokinesis, while strain COH1 appeared to principally interact with the matrix material seen surrounding the VECs (Figure 3-3). Importantly, VEC monolayers remained confluent after incubation with the bacteria (discussed further in sub-section 1.9).

It was not feasible to work with all five GBS strains throughout this project. As such, strains NEM316, 515 and COH1 were chosen for further studies. This selection displayed a range of capacities for coaggregation with *C. albicans* and association with VECs, while also representing key capsular serotypes. Strains NEM316 and COH1 are capsular serotype III, while 515 is serotype Ia, these being two of the three most common serotypes associated with severe, invasive disease (Zhang et al., 2006;Jiang et al., 2008;Farley, 2001).

To optimise the VEC assay for future work, the effect of incubation period on the number of associating bacteria was tested using GBS strain NEM316. Figure 3-4 shows the number of NEM316 bacteria associating with VECs at 30 minutes, 60 minutes and 120 minutes post-incubation. There was more than a quadrupling in the number of bacteria associating with the VECs between 60 min ($2.9x10^5$ CFU/ml) and 120 min ($2x10^6$ CFU/ml). To minimise potential issues with cytotoxicity, particularly when introducing a second microorganism (*C. albicans*) in subsequent work, a 1 h timepoint was selected for GBS incubation, unless otherwise stated.

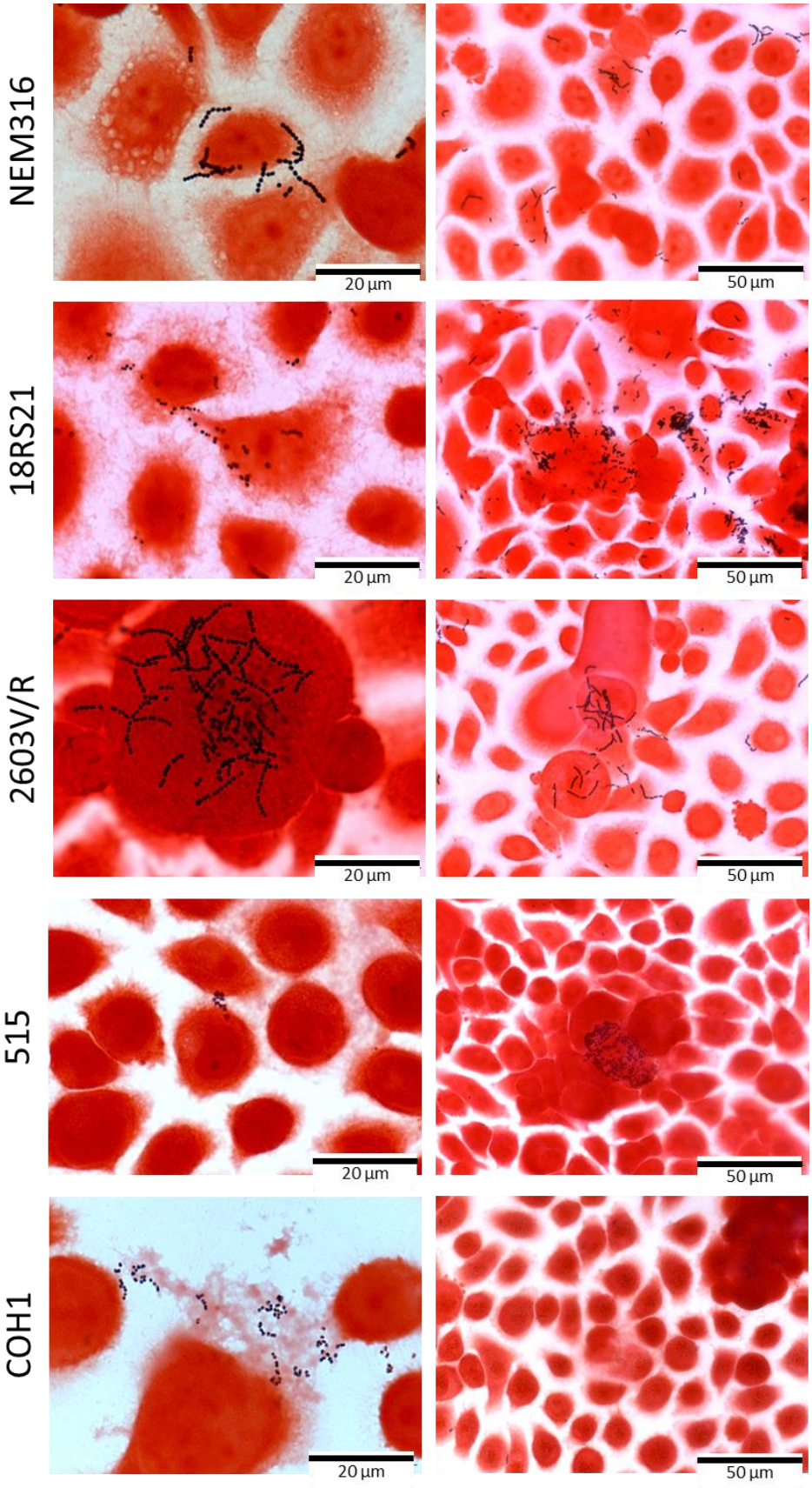

Figure 3-3 Representative brightfield micrographs of VECs incubated with GBS strains.

VEC monolayers were grown on 19 mm sterile glass coverslips and incubated with GBS suspensions (MOI 2.5) for 2 h. Coverslips were then Gram stained, heat fixed and mounted onto a glass slide.

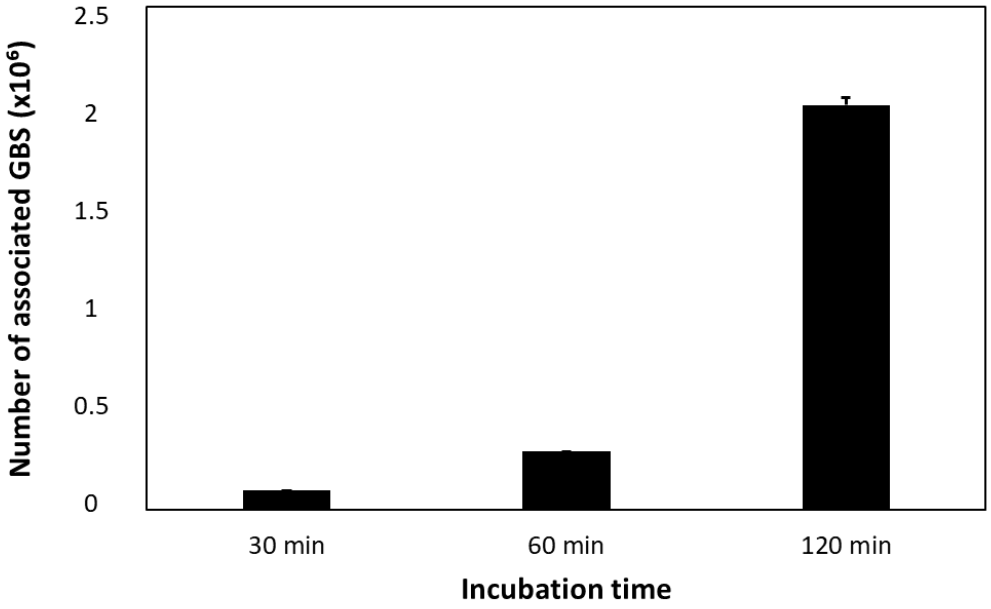

Figure 3-4 Effects of incubation time on association of GBS with VECs.

VEC monolayers were incubated with GBS suspensions (MOI 2.5) for either 30 minutes, 60 min or 120 min, as indicated. Monolayers were then lysed and numbers of associated GBS enumerated by viable count. Data are presented as mean ± SD, n=1.

3.2.3 Testing influence of *C. albicans* hyphae formation on GBS association with VECs

Hyphae tropism had been described for a number of bacteria when interacting with *C. albicans*, including *S. gordonii* and *S. aureus* (Peters et al., 2010;Silverman et al., 2010). Moreover, GBS was found to preferentially bind to *C. albicans* hyphae rather than blastospores in the coaggregation studies (Figure 3-1A). With the aim of developing a dual-species microbial association assay, a pilot study was performed to compare the effects of adding GBS and *C. albicans* to VECs at the same time, compared to 'priming' *C. albicans* on the VECs first to allow hyphae formation. To investigate this, microbes were either added to the VECs together and incubated for 1 h, or *C. albicans* was pre-incubated for 1 h with VECs before GBS was added and incubated for a further 1 h (Figure 3-5). The number of NEM316 bacteria recovered from VEC monolayers was 39% lower at $1.7x10^5$ CFU/mL when incubated with *C. albicans* for 1 h compared to the monospecies control ($2.8x10^5$ CFU/mL) (Figure 3-5). By contrast, when *C. albicans* was pre-incubated with the VECs for 1 h before NEM316 was added and incubated for a further 1 h, numbers of associated NEM316 were increased by 62% to $4.5x10^5$ CFU/mL relative to monospecies control (Figure 3-5). It was hypothesised that this increase was

due to *C. albicans* formation of hyphae during the initial 1 h period providing additional targets for GBS to interact with.

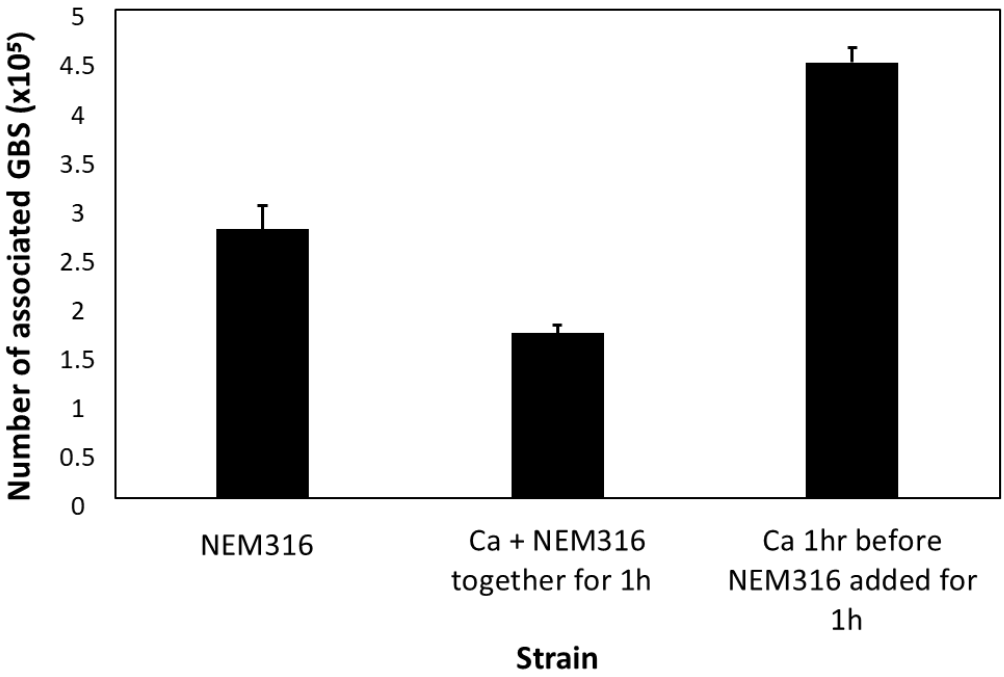

Figure 3-5 Effect of *C. albicans* pre-incubation on GBS association with VECs.

VECs were incubated with either GBS (MOI 2.5) for 1 h, *C. albicans* (MOI 2.5) and GBS for 1 h, or *C. albicans* for 1 h followed by GBS for 1 h. Monolayers were lysed and numbers of associated GBS were enumerated by viable count. Data are presented as mean ± SD, n=1.

C. albicans had been previously tested for the ability to form hyphae in K-SFM tissue culture medium (data not shown). To confirm the ability of *C. albicans* to form hyphae when incubated with VECs, the experiment was repeated using VECs grown on glass cover slips that were Gram stained after incubation with *C. albicans* and NEM316. Hyphae were visibly longer when *C. albicans* was incubated with VECs for 2 h compared to 1 h (Figure 3-6, rows 1-2). Furthermore, these differences in hyphal length were not affected by the presence of GBS (compare Figure 3-6, row 1 with 3, row 2 with 4), implying that GBS NEM316 did not inhibit the ability of *C. albicans* to form hyphae. Thus, it was concluded that *C. albicans* initiates hyphae formation after approximately 1 h incubation with VECs. Furthermore, the presence of *C. albicans* hyphae may enhance the association of GBS NEM316 with VECs.

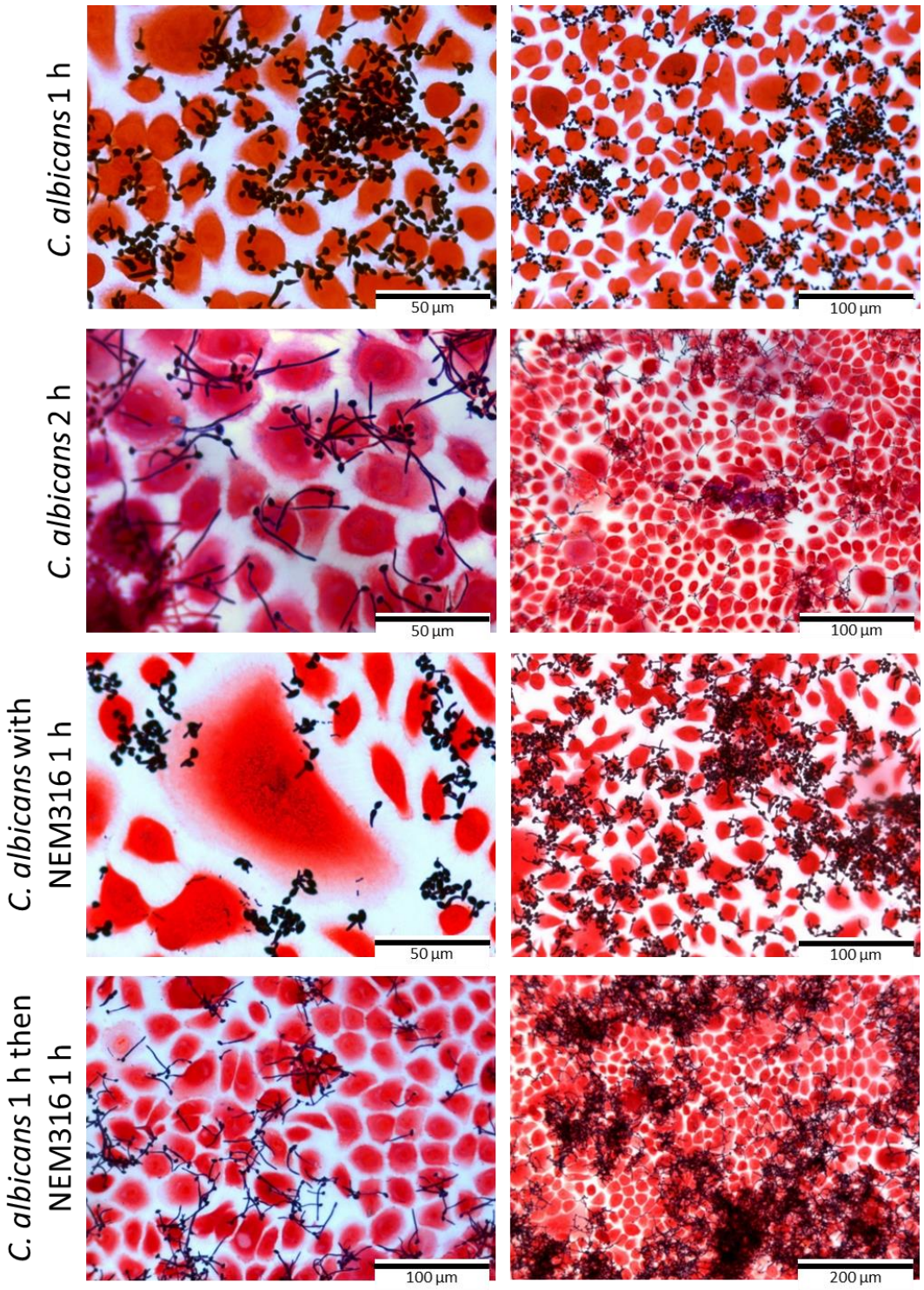

Figure 3-6 Representative brightfield micrographs of VECs incubated with *C. albicans* and NEM316.

VEC monolayers were grown on 19 mm sterile glass coverslips, and incubated with *C. albicans* suspensions (MOI 2.5) for 1 or 2 h, or with *C. albicans* and NEM316 (MOI 2.5) for 1 h, or with *C. albicans* for 1 h before addition of NEM316 and incubation for a further 1 h. Coverslips were then Gram stained, heat fixed and mounted onto a glass slide.

To confirm that the effects observed in Figure 3-5 were not restricted to GBS strain NEM316, the experiment was repeated using GBS strains 515 and COH1 alongside NEM316. No significant difference in GBS association was observed for any of the strains when *C. albicans* was added at the same time as GBS compared to monospecies controls (Figure 3-7, grey bars). However, each GBS strain was found to significantly increase in association with VECs when added 1 h after *C. albicans* (Figure 3-7, black bars). NEM316 was promoted by 2.9-fold, 515 by 3.1-fold and COH1 by 4.5-fold.

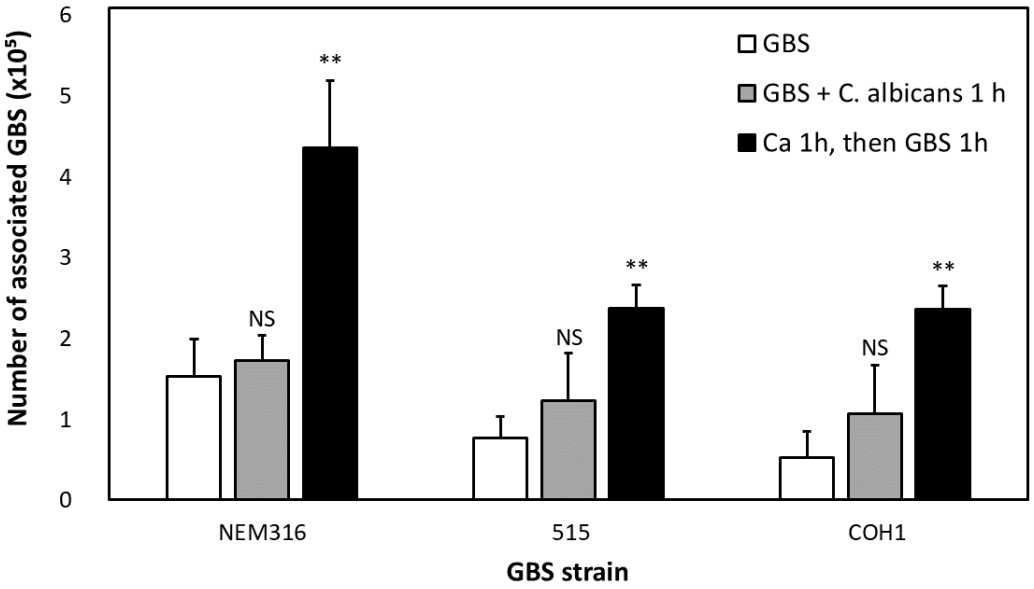

Figure 3-7 Effects of *C. albicans* on association of GBS with VECs.

VEC monolayers were incubated with GBS suspensions (MOI 2.5) for 1 h (white bars) or with *C. albicans* (MOI 2.5) and GBS suspensions for 1 h (grey bars), or with *C. albicans* for 1 h followed by GBS for a further 1 h (black bars). Monolayers were then lysed and numbers of associated GBS enumerated by viable count. ** $P<0.01$, NS $P>0.01$ when compared against the monospecies controls, as determined by unpaired Student's t-test with Bonferroni correction. Data are presented as mean ± SD, n=4.

These data indicated an intriguing potential relationship between *C. albicans* and GBS, which became the focus of subsequent studies. Thus, for all future experiments, *C. albicans* was pre-incubated with the VECs for 1 h before addition of bacteria.

At this point, the COH1 GBS strain was exchanged. This was due to a personal communication from Dr Kelly Doran (San Diego State University) in which it was indicated that the COH1 strain should interact strongly with VECs. Dr Doran kindly gifted a stock of COH1 (designated unique reference number UB2866), and this was found to associate with VECs in higher numbers than seen for the previous stock. Importantly,

however, this new stock interacted with *C. albicans* similarly to the previous version i.e. the stock coaggregated at only low levels yet was promoted in association with VECs by *C. albicans* (data not shown).

3.2.4 Confocal micrographs of GBS and *C. albicans* interactions with VECs at 2 h

In an effort to verify the apparent enhancement of GBS association with VECs by *C. albicans* as determined by viable count data, confocal micrographs of the VECs incubated with *C. albicans* and GBS were taken. VECs were grown to confluence on 19 mm glass coverslips before incubation with *C. albicans* for 1 h, after which GBS strains NEM316, 515 or COH1 were added and monolayers were incubated for a further 1 h. *C. albicans* was labelled with Calcofluor White (blue), while the VEC actin was stained with phalloidin-TRITC (red) and GBS was immunolabelled with an Alexafluor-488 antibody (green). For NEM316, a visible increase in the number of bacteria was observed when incubated with *C. albicans* compared to monospecies controls (Figure 3-8). This was not as obvious for strains 515 or COH1. However, biovolume (μm^3) data for GBS was also collected as a semi-quantification method using the Volocity® software and Imaris® v7.5 software. NEM316 biovolume rose by 1.8-fold, increasing from 936 μm^3 in the monospecies to 1748 μm^3 when incubated with *C. albicans,* while 515 biovolume increased by 3.8-fold from 118 μm^3 to 449 μm^3. COH1 biovolume increased by 8.9-fold, rising from 85 μm^3 to 761 μm^3. This demonstrated that for all three GBS strains, there was a clear increase in the number of fluorescently-stained bacteria when co-incubated with *C. albicans* on VECs than when incubated alone (Figure 3-9). This supported the viable count data from Figure 3-7.

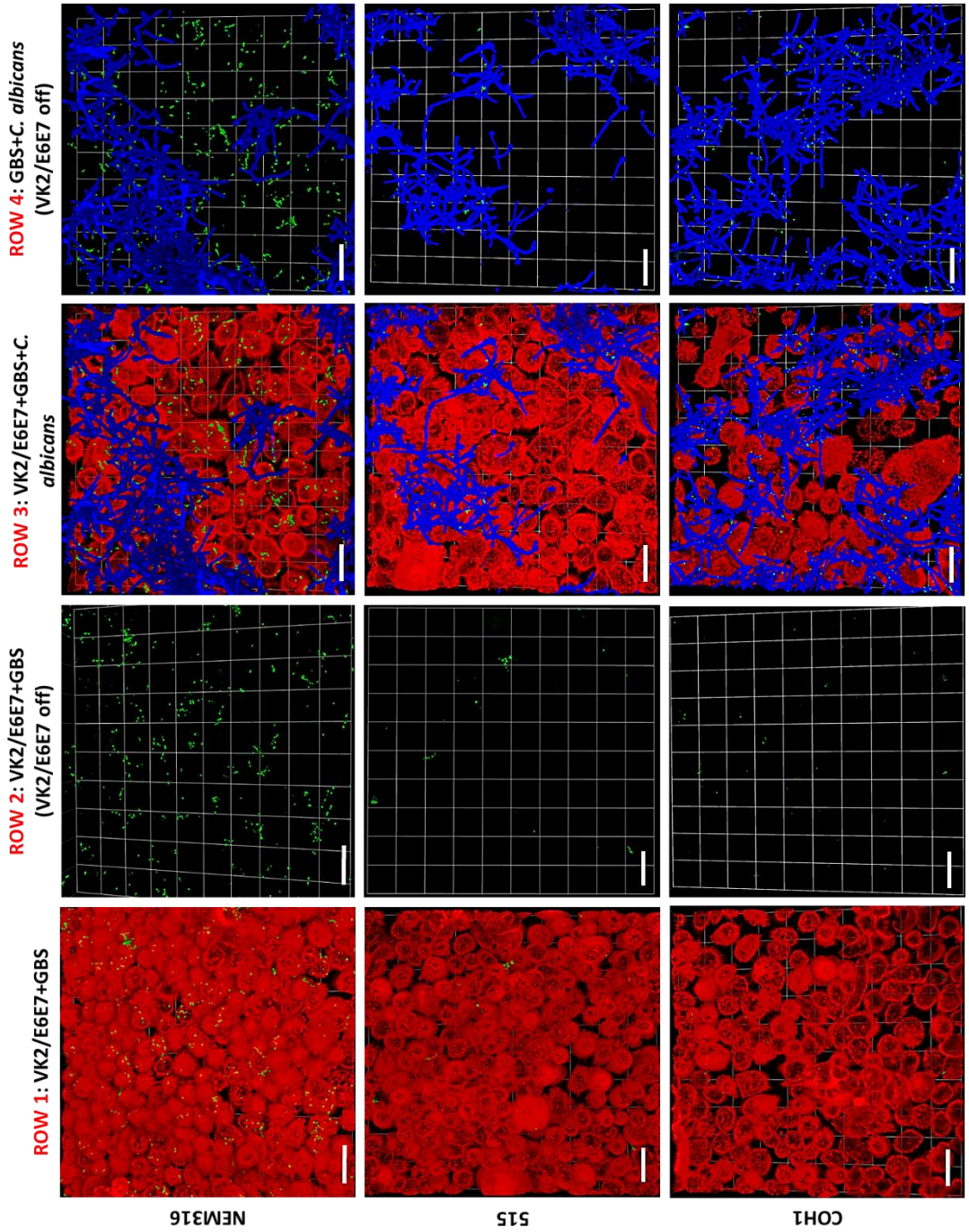

Figure 3-8 Representative confocal micrographs of *C. albicans*-GBS association with VECs.

VEC monolayers were incubated with GBS (MOI 25) alone for 1 h (rows 1 and 2) or with *C. albicans* (MOI 2.5) for 1 h followed by GBS for a further 1 h (rows 3 and 4). Cells were then fixed, stained and mounted onto glass slides. GBS was labelled using Alexafluor-488-conjugated antibody (green), while *C. albicans* was labelled with Calcofluor White (blue), and VEC cells were labelled with phalloidin-TRITC (red). GBS strains NEM316 (top panels), 515 (middle panels) and COH1 (bottom panels) were tested. Rows 2 and 4 are duplicates of rows 1 and 3, respectively, in which the red filter (i.e. the VECs) has been removed. Scale bars, 100 µm.

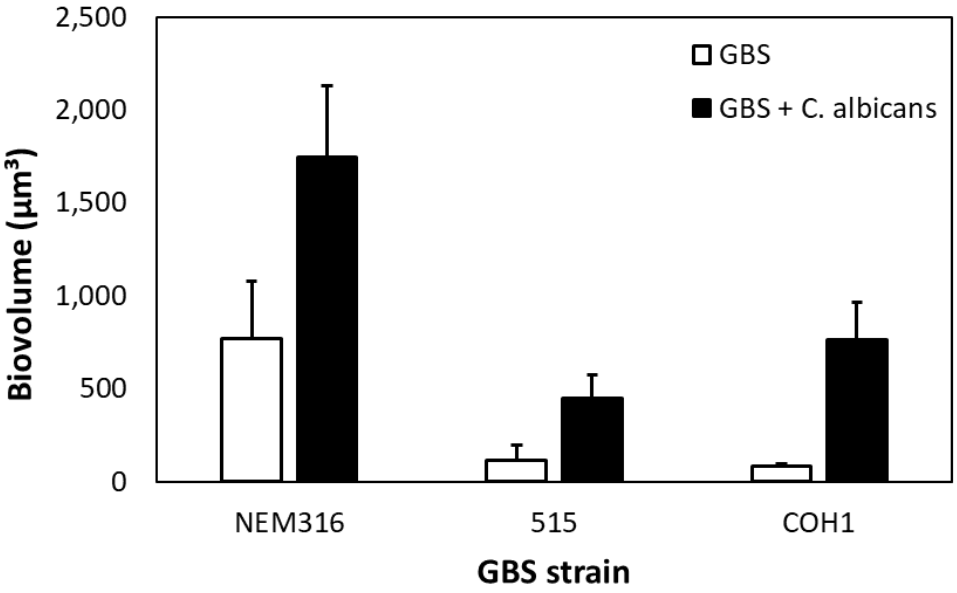

Figure 3-9 Semi-quantification of GBS from confocal micrographs illustrated in Figure 3-8.

Images were processed using Volocity® software and Imaris® software was used to calculate GBS biovolumes (µm³). White bars represent biovolume of GBS from monospecies images, while black bars show biovolume of GBS from dual-species images. Data are presented as mean ± SD, n=1.

3.2.5 Invasion studies

All studies thus far determined the number of associated microbes, which represents both the number of adherent bacteria plus any internalised bacteria. A hallmark of pathogenesis for these microorganisms is their ability to invade host tissues. Furthermore, it has been shown that invasion of epithelia by *S. aureus* can be facilitated by binding *C. albicans* hyphae and subsequently 'piggy-backing' into host cells (Peters et al., 2012). As such, a variation of the association assay was conducted to monitor the number of internalised GBS in the presence or absence of *C. albicans*. For these studies *C. albicans* was incubated with VEC monolayers for 1 h, followed by GBS addition and incubation for a further 1 h, as before. Monolayers were then exposed to media containing antibiotics for 2 h to kill any extracellular GBS. VECs were lysed and recovered GBS represented those cells that had been internalised within VECs and thus protected from the antibiotics.

There was a significant (2.8-fold) decrease in the number of internalised NEM316 when in the presence of *C. albicans* compared to monospecies infection (Figure 3-10). This was somewhat surprising, as one might have predicted that the increase in numbers of associated GBS in the presence of *C. albicans* seen in Figure 3-7 would include

internalised bacteria. It was hypothesised that GBS were preferentially binding *C. albicans* hyphae over VECs, which offered the potential for 'piggy-backing' into the VECs, but that 2 h may have been too early for *C. albicans* hyphae to have initiated invasion. To test this theory, the association/invasion assay was extended to 6 h (1 h *C. albicans* + 5 h GBS).

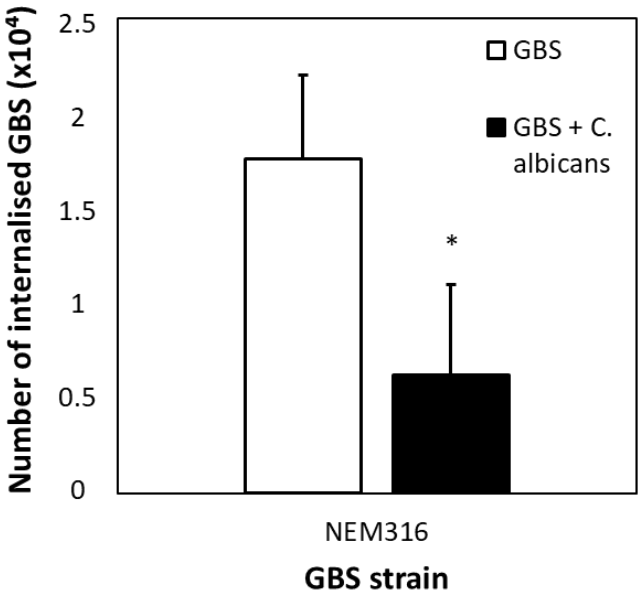

Figure 3-10 Effects of *C. albicans* on GBS invasion of VECs.

VECs were incubated with *C. albicans* (MOI 2.5) for 1 h before addition of GBS strain NEM316 (MOI 25) and incubation for a further 1 h. Epithelial cell monolayers were then exposed to 200 µg/mL gentamicin and 10 µg/mL penicillin for 2 h, lysed and numbers of internalised GBS enumerated by viable count. Data are presented as mean ± SD,* P<0.05 when compared against the monospecies control as determined by Student's t test; n=3.

Under these conditions, numbers of associated bacteria were significantly promoted in the presence of *C. albicans* for all three GBS strains when compared against monospecies controls (Figure 3-11A). This was similar to the effect seen after 1 h incubation (Figure 3-7), but rather than the 2.9-fold increase observed in Figure 3-7 when *C. albicans* was present, NEM316 was 5.8-fold promoted, while for 515 this was 39.4-fold and for COH1 this was 14.7-fold. The corresponding numbers of internalised GBS are represented in Figure 3-11B. Unlike the reduced levels of invasion shown in Figure 3-10, numbers of internalised NEM316 and COH1 were both significantly higher in the presence of *C. albicans* than for the corresponding monospecies infection. Invasion levels were enhanced by 6.6-fold for NEM316 and by 3.2-fold for COH1 (Figure

3-11B). By contrast, 515 invasion levels were significantly lower in the presence of *C. albicans*, amounting to a 6-fold decrease in invasion relative to the monospecies control (Figure 3-11B).

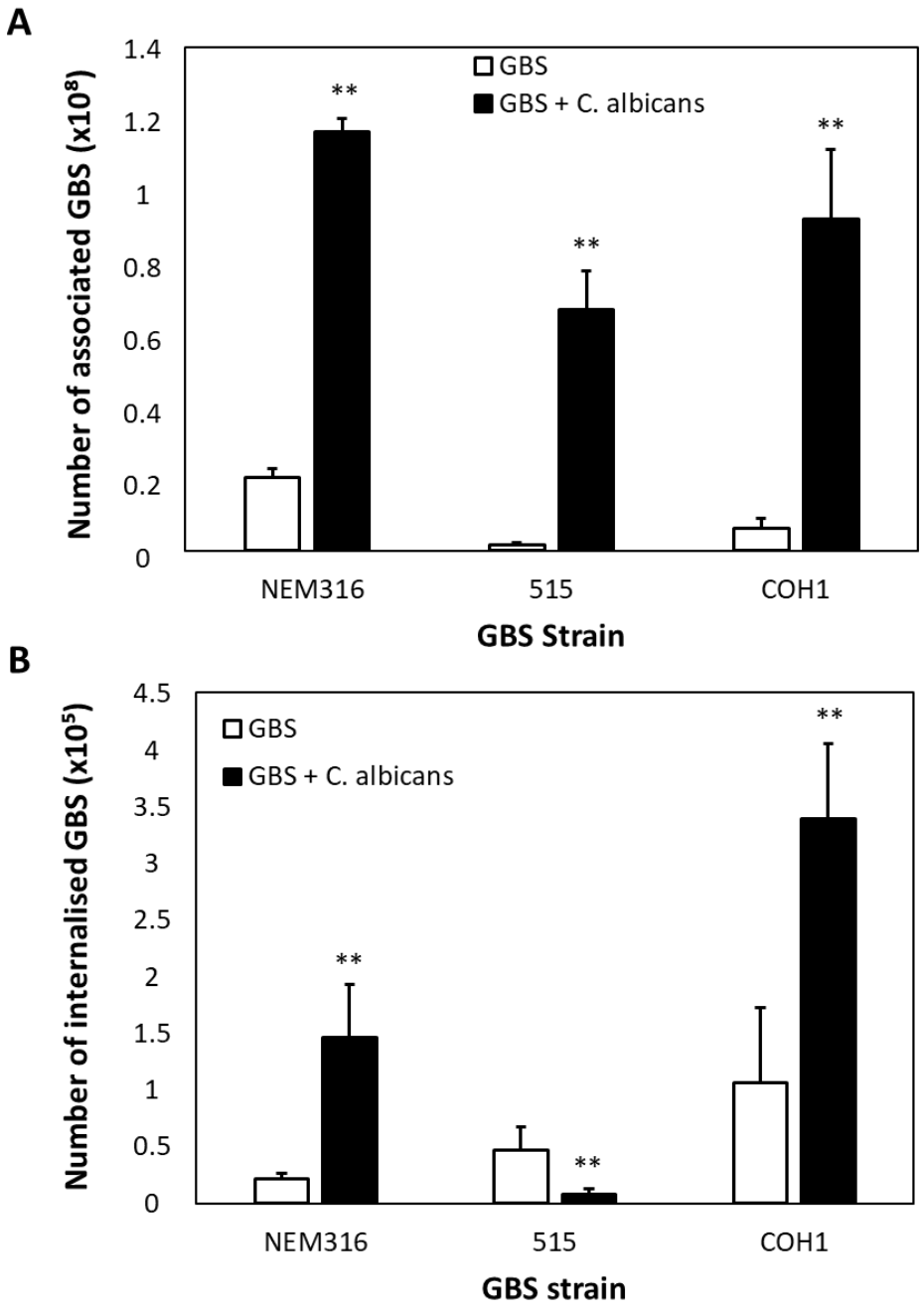

Figure 3-11 Effects of *C. albicans* on GBS association (A) and invasion (B) of VECs.

VECs were incubated with *C. albicans* (MOI 2.5) for 1 h before addition of GBS (MOI 25) and incubation for a further 5 h. For invasion studies, epithelial cell monolayers were then exposed to 200 µg/mL gentamicin and 10 µg/mL penicillin for 2 h, lysed and numbers of internalised GBS enumerated by viable count. ** $P<0.01$ when compared to the monospecies control, as determined by unpaired Student's t-test. Data are presented as mean ± SD, n=4.

3.2.6 Confocal micrographs of GBS and *C. albicans* interactions with VECs at 6 h

To further validate the viable count data, the confocal microscopy studies detailed in section 3.2.4 were repeated, with the modification that the incubation period with GBS was extended by 4 h, i.e. VECs were incubated with *C. albicans* for 1 h before GBS strains NEM316, 515 or COH1 were added and monolayers were incubated for a further 5 h. In contrast to the equivalent 2 h studies (Figure 3-8), there was a visible increase in the number of GBS present with *C. albicans* for all three GBS strains compared to the monospecies controls (Figure 3-12). This was particularly striking with NEM316 (Figure 3-12, top panels) and was supported by corresponding biovolume data (Figure 3-13). NEM316 biovolume rose by 5.6-fold, increasing from 474 μm^3 in the monospecies control to 2677 μm^3 when incubated with *C. albicans,* while 515 biovolume increased by 3.8-fold from 114 μm^3 to 435 μm^3. COH1 biovolume increased by 103-fold, rising from 19 μm^3 to 1996 μm^3. Unfortunately, GBS strain COH1 (Figure 3-12, bottom panel) did not stain well with the anti-GBS primary antibody, possibly due to the fact that this strain is known to have a particularly thick capsule (Rubens et al., 1993;Tissi et al., 1998). This lack of staining likely accounts for the apparent lower numbers/biovolume of COH1 compared to NEM316, contrary to the quantitative data represented in Figure 3-13.

Of note, GBS was seen to be interacting with *C. albicans* hyphae (as indicated by white arrows), but there was also an increase in the numbers of bacteria interacting with areas of the epithelium that were not seemingly colonised by *C. albicans* (as indicated by red arrows). This suggested that direct physical interactions between GBS and *C. albicans* may not wholly explain the capacity for *C. albicans* to promote GBS association with VECs.

Although there were examples from the confocal micrographs where *C. albicans* hyphae appeared to have invaded VECs, it could not be clearly established whether GBS were intracellular or extracellular. Efforts were made to establish a confocal assay which used differential antibody staining, so that intracellular GBS would be stained a different colour to extracellular bacteria. Unfortunately, however, these attempts were unsuccessful.

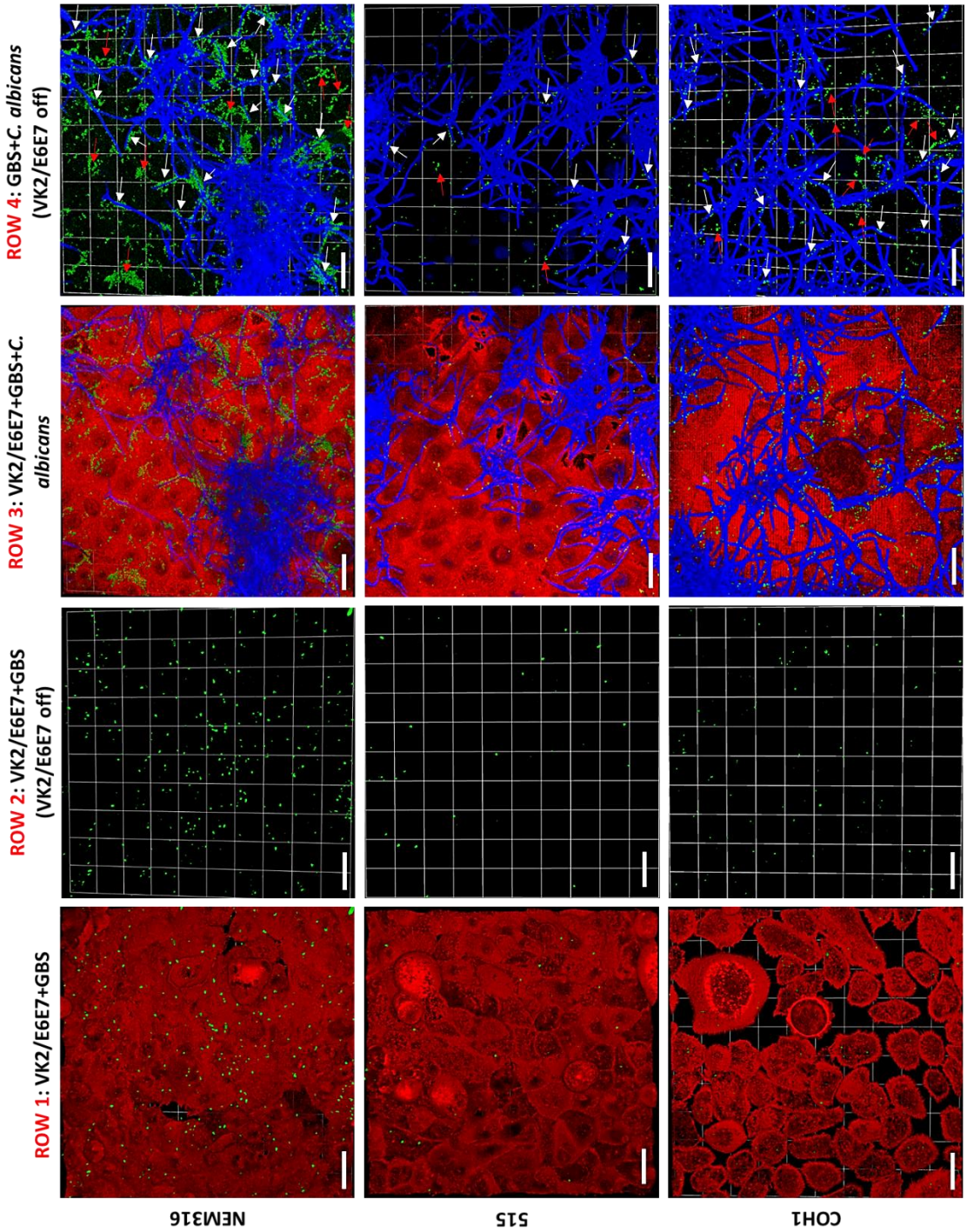

Figure 3-12 Representative confocal micrographs of *C. albicans*-GBS association with VECs.

VEC monolayers were incubated with GBS (MOI 25) alone for 5 h (rows 1 and 2) or with *C. albicans* (MOI 2.5) for 1 h followed by GBS for a further 5 h (rows 3 and 4). Cells were then fixed, stained and mounted onto glass slides. GBS was labelled using Alexafluor-488-conjugated antibody (green), while *C. albicans* was labelled with Calcofluor White (blue), and VECs were labelled with phalloidin-TRITC (red). GBS strains NEM316 (top panels), 515 (middle panels) and COH1 (bottom panels) were tested. Rows 2 and 4 are duplicates of rows 1 and 3, respectively, in which the red filter (i.e. the VECs) has been removed. White arrows indicate areas where GBS is bound to *C. albicans* hyphae, while red arrows indicate areas where GBS is found in the absence of *C. albicans*. Scale bars; 100 µm.

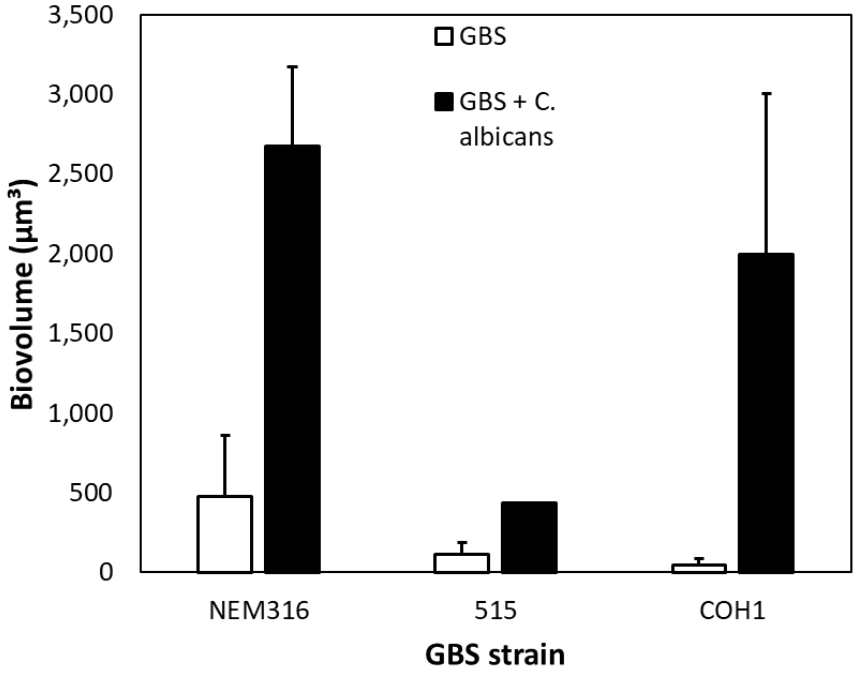

Figure 3-13 Quantification of GBS from confocal micrographs illustrated in Figure 3-12.

Images were processed using Volocity® software and Imaris® software was used to calculate GBS biovolumes (µm³). White bars represent biovolume of GBS from monospecies images, while black bars show biovolume of GBS from dual-species images. Data are presented as mean ± SD, n=1.

3.2.7 Association of *C. albicans* with VECs and GBS

Having demonstrated that *C. albicans* could enhance association of GBS with VECs, it was of interest to investigate if this effect was reciprocal. This was assessed using the experiment described earlier to determine numbers of GBS, with *C. albicans* quantified instead. As such, levels of *C. albicans* associated with VECs were determined in the presence or absence of GBS. Elevated numbers of *C. albicans* were recovered from the VECs for all three GBS strains (Figure 3-14). Furthermore, levels of enhancement were comparable (4.6-fold by NEM316, 4-fold by 515, 4.5-fold by COH1), despite the varying capabilities that these GBS strains exhibited in their interactions with VECs in monospecies infections (Figure 3-7).

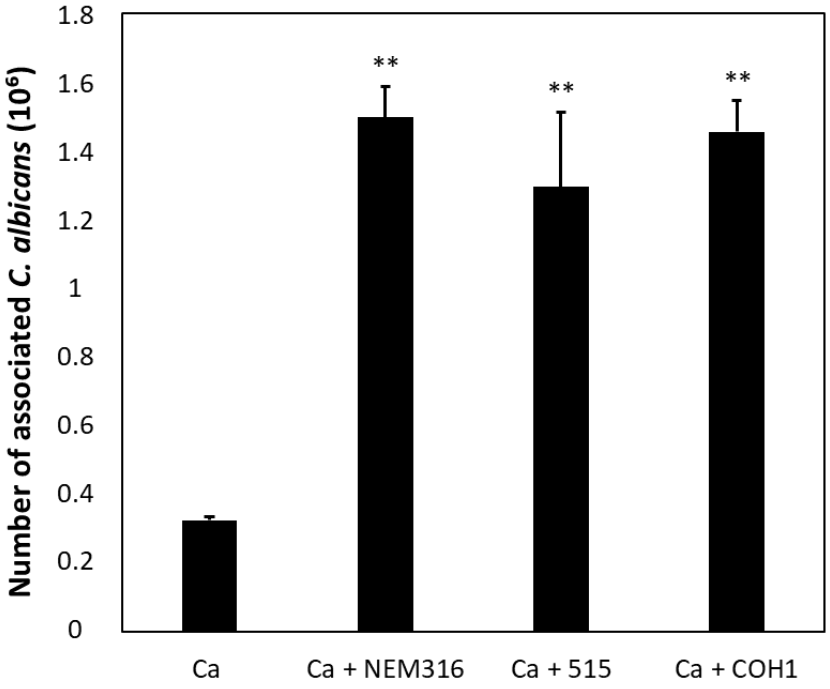

Figure 3-14 Effects of GBS on *C. albicans* association with VECs.

VEC monolayers were incubated with *C. albicans* (MOI 2.5) for 1 h before addition of GBS (MOI 2.5) and incubation for a further 1 h. Monolayers were then lysed and numbers of associated *C. albicans* enumerated by viable count. Data are presented as mean ± SD,** P<0.01 when compared against the monospecies control as determined by unpaired Student's t-test with Bonferroni correction; n=3.

Taken together, these data suggested a synergistic relationship between *C. albicans* and GBS, in which coassociation between these two microorganisms facilitated interactions of both species with vaginal epithelium.

3.2.8 VEC viability during microbial association assays

Integrity of the VEC monolayers was critical to correct interpretation of the data for the assays performed in this chapter, as otherwise the data could simply reflect the capacity for the microbes to adhere to the polystyrene microtitre plate. To ensure the monolayers were maintained during the microbial association assays presented here, every time a new assay was devised, brightfield images were taken of the cell monolayer at each step (Figure 3-15). This confirmed that VEC monolayers were retained following mono- or dual-species infections after both 2 h and 6 h incubation periods. Only following trypsin incubation and subsequent water washes was VEC lysis apparent, as

would be expected. The appearance of the VECs also gave some indication as to their apparent 'health'. After 1 h GBS incubation and after 2 h or 6 h *C. albicans* incubation (Figure 3-15), VECs were similar in appearance to monolayers at the start of the assay. However, VECs incubated with NEM316 for 5 h +/-*C. albicans* for 1 h appeared less healthy. This likely reflected the fact that a higher MOI for GBS was used for the invasion assays, due to the fact that a much lower number of bacteria were internalised compared to numbers of adherent bacteria. This higher MOI could be expected to have greater cytotoxic effects.

To further assess the cytotoxic effects of the mono- and dual-species infections on VECs, an LDH assay was used. The percentage cytotoxicity was calculated as experimental LDH detected over maximum LDH release (as determined following complete VEC lysis) and multiplied by 100. After 2 h (association assay), there was no major change in LDH levels for any of the strain combinations compared to the VEC-only control level of 5.7% (Figure 3-16A). Conversely, at 6 h post-infection (Figure 3-16B), there was a slight increase in cytotoxicity to 25.8% for VECs incubated with just *C. albicans*, although this was not significant. When VECs were incubated with only GBS (MOI 2.5), there was a slight increase in cytotoxicity with all three strains (to 23.3% for NEM316, 10.4% for 515, 12.1% for COH1), but this was not significant. There was a steep and significant increase in cytotoxicity for cells incubated with both microorganisms, rising to 84.8% for *C. albicans* with NEM316, 84.1% with 515 and 62% with COH1. The high cytotoxicity of VECs exposed to both *C. albicans* and GBS over 6 h corroborates their 'unhealthy' appearance at this time point (Figure 3-12), and there is a distinct difference in cell morphology which can be observed when the side view of images taken at 2 h versus 6 h are compared (Figure 3-17).

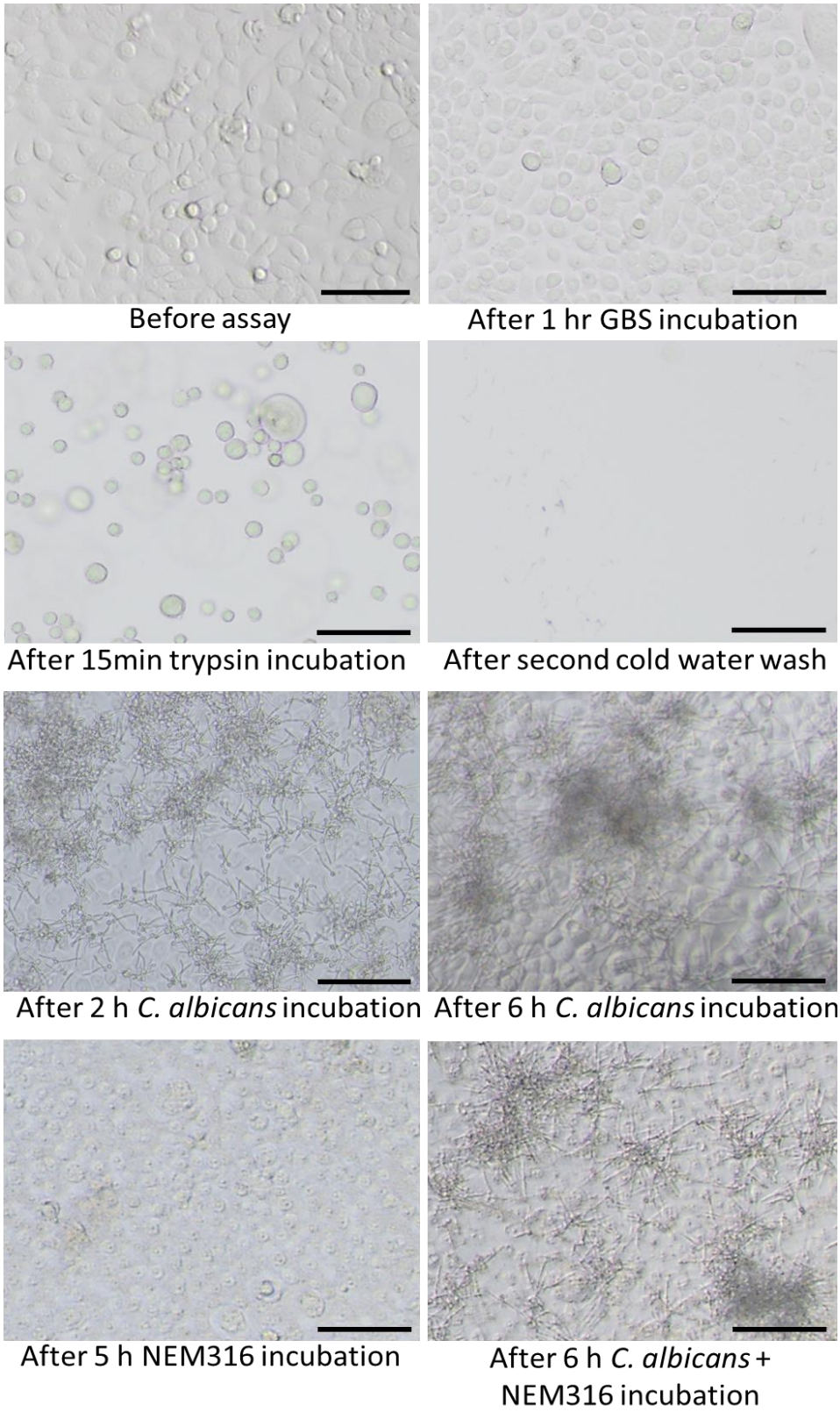

Figure 3-15 Representative brightfield images of VEC monolayers at each stage of microbial association assays.

From top left to right: before an assay, after 1 h GBS incubation (MOI 2.5); after 1 h GBS incubation (MOI 2.5) followed by PBS washes and 15 min trypsin incubation; after two 500 µL cold water washes at the end of an assay; after 2 h incubation with *C. albicans* (MOI 2.5); after 6 h incubation with *C. albicans* (MOI 2.5); after 5 h incubation with NEM316 (MOI 25); after 1 h *C. albicans* incubation (MOI 2.5) followed by 5 h incubation with NEM316 (MOI 25). Scale bars; 100 µm.

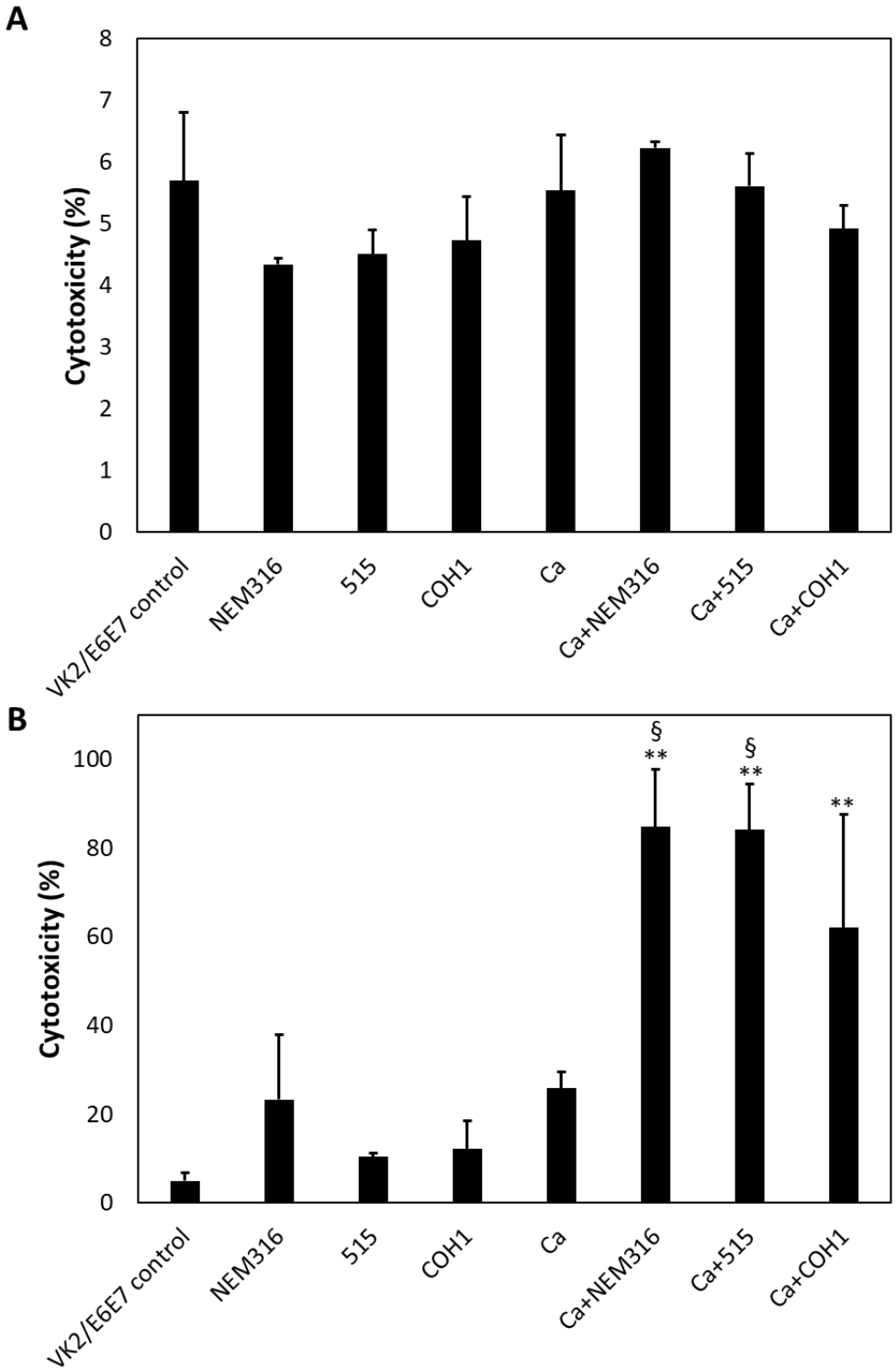

Figure 3-16 Percentage cytotoxicity of VEC monolayers after microbial association assays.

Supernatants were collected at the end of association assays and tested for LDH. Percentage cytotoxicity was calculated as experimental LDH release (OD_{490}) divided by maximum LDH release (OD_{490}) and multiplied by 100. A) Percentage cytotoxicity of VEC monolayers in response to incubation with *C. albicans* (MOI 2.5) for 2 h, GBS strains NEM316, 515 or COH1 (MOI 2.5) for 1 h, or *C. albicans* for 1 h followed by GBS for a further 1 h; n=1. B) Percentage cytotoxicity of VEC monolayers in response to incubation with *C. albicans* (MOI 2.5) for 6 h, GBS strains NEM316, 515 or COH1 (MOI 2.5) for 5 h, or with *C. albicans* for 1 h followed by 5 h with GBS. ** indicates $P<0.01$ when compared to VEC control, § indicates $P<0.01$ when compared to monospecies GBS or *C. albicans*, as calculated by one-way ANOVA with Tukey post-test. Data are presented as mean ± SD, n=4.

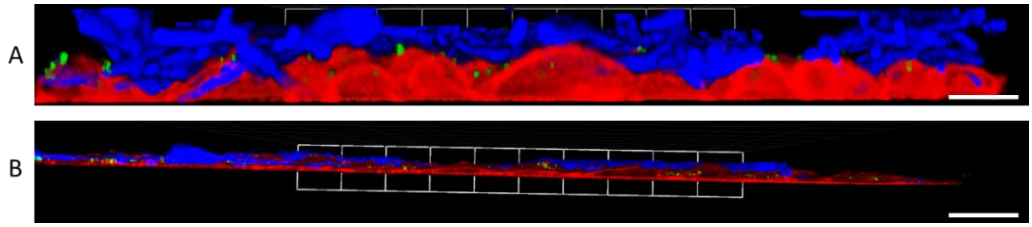

Figure 3-17 Comparison of confocal micrographs.

A) Side view of VEC+*C. albicans*+NEM316 from Figure 3-8, i.e. 2 h post-infection. B) Side view of VEC+*C. albicans*+NEM316 from Figure 3-12, i.e. 6 h post-infection. Scale; 100 µm.

3.3 Discussion

The studies presented in this chapter sought to investigate the interactions of GBS with *C. albicans*, and the capacity for any such interactions to modulate association with VECs. Under planktonic conditions, all five strains of GBS tested were able to coaggregate with *C. albicans*, albeit to variable levels. Moreover, all five GBS strains were observed to exhibit a tropism for *C. albicans* hyphae rather than blastospores, as has been described for other bacteria, including streptococci such as *S. gordonii*, *S. oralis* and *S. sanguinis* (Jenkinson et al., 1990;Silverman et al., 2010;Bamford et al., 2009). This implies that GBS and *C. albicans* are coaggregation partners, although the precise nature of this partnership, at least for GBS, is clearly strain-dependent. The extent to which different GBS strains coaggregated with *C. albicans* did not correlate with capsular serotype, suggesting that capsular polysaccharide does not directly mediate the coaggregation interactions. Nonetheless, levels of CPS expression may impact coaggregation capacity via potential masking of the GBS receptor(s) targeted by *C. albicans*. Indeed, GBS strain COH1, which exhibited the lowest levels of coaggregation of the five GBS strains tested, is known to produce a particularly thick capsule (Rubens et al., 1993;Tissi et al., 1998). Alternatively, differences in the coaggregation profiles of the GBS strains may reflect variations in the expression levels of another surface-expressed determinant(s). Despite variable levels of coaggregation, the fact that all five GBS strains preferentially bound candidal hyphae suggested that GBS strains may target a common hyphal receptor. This was investigated and is presented in Chapter 4.

The tropism of GBS for *C. albicans* hyphae was also seen in the context of these microorganisms associating with VECs. When *C. albicans* blastospores and GBS strains NEM316, 515 or COH1 were added to VEC monolayers simultaneously, comparable

numbers of GBS cells were recovered relative to monospecies control. This implies that blastospores and GBS cells were not competing for VEC binding sites, and thus suggests that either these microorganisms target different VEC receptors, or that receptor availability was of sufficient abundance to support association by both microbes. By contrast, the presence of candidal hyphal filaments that had already associated with VEC monolayers resulted in enhanced association levels by all three GBS strains. Coaggregation studies had indicated the capacity for GBS to bind directly to *C. albicans* hyphae. One likely explanation for these effects was therefore that *C. albicans* hyphae provided additional binding sites to GBS alongside the VECs. In support of this, confocal micrographs showed GBS cells bound to *C. albicans* hyphae, and with increasing incubation period and thus extent of hyphal filamentation, so the interactions between GBS and candidal hyphae became more apparent, and the scale of enhanced GBS association with VECs in the presence of *C. albicans* more pronounced. Genes controlling hyphae formation were found to be upregulated when *C. albicans* was grown in the presence of *S. gordonii* (Dutton et al., 2016). If a similar effect was mediated by GBS, this could further enhance GBS interactions with VECs. However, while not quantified directly, there was no indication that GBS affected fungal morphology.

Alongside the direct physical *C. albicans*-GBS interactions in the presence of VECs, there was also a significant increase in the number of bacteria binding VECs in areas where *C. albicans* was not present. This raised the possibility that *C. albicans* hyphae might engage with the vaginal epithelium in a way that made the VECs more permissive to GBS association. Such an indirect effect could explain why VEC association by GBS strain COH1 was significantly elevated in the presence of *C. albicans*, despite the fact that COH1 exhibited only a low level of coaggregation with *C. albicans* under planktonic conditions. The potential for indirect effects between *C. albicans* and VECs to modulate GBS association levels was investigated further and is presented in Chapter 4.

Similar to the effects of *C. albicans* on VEC association by GBS, GBS was also shown to significantly promote association of *C. albicans* with VECs. Again, given the coaggregation capabilities of these two microbes, it is possible that such effects reflect the capacity for bound GBS to directly serve as additional binding sites for candidal hyphal filaments. However, while the three GBS strains tested exhibited variable levels of VEC association when incubated alone, *C. albicans* was promoted to similar levels of

association regardless of GBS strain. This might suggest that GBS mediates its effects on *C. albicans* via an indirect mechanism, as discussed above.

It is worth noting that the conditions used for the planktonic coaggregation assays and VEC association assays were different. Thus, it cannot be ruled out that the surface receptor profile of *C. albicans* and/or GBS changed following binding to VECs or in response to the different media, which in turn impacted *C. albicans* interactions with GBS. For example, glucose concentration was found to affect the expression of a number of GBS genes, including those controlling expression of virulence factor BibA (Di Palo et al., 2013), while GBS virulence genes encoding pili and BibA were differentially expressed in relation to pH (Santi et al., 2009). Likewise, the secreted aspartyl proteinases (SAPs) of *C. albicans* have been found to be differentially expressed in response to pH and presence of amino acids (Hube et al., 1994), while phospholipase B was expressed when *C. albicans* was grown in rich media at 30 °C but not 37 °C (Mukherjee et al., 2003). Expression of different surface molecules was hypothesised to be the reason that there was an increase in coaggregation between *C. albicans* and *S. gordonii* or *S. sanguinis* under starvation conditions (Jenkinson et al., 1990). Further investigations were performed to explore the coordinated responses of *C. albicans*, GBS and VECs. These data are presented in Chapter 5.

C. albicans has been shown to facilitate invasion of oral epithelium by *S. aureus* (Schlecht et al., 2015a). *S. aureus* bacteria bind to the *C. albicans* hyphae, and as the hyphae invade oral epithelium, *S. aureus* cells are carried with them. Since GBS was also observed to exhibit a tropism for *C. albicans* hyphae, it was possible that a similar 'piggy-back' mechanism was occurring for GBS invasion of VECs. As such, it was originally assumed that elevated numbers of GBS associated with VECs in the presence of *C. albicans* may correspond to both externally bound and internalised bacteria. However, this was not seen at 2 h and, in fact, levels of internalised GBS NEM316 were reduced in the presence of *C. albicans*. One potential explanation for this observation was that GBS was preferentially binding to *C. albicans* over VECs. However, this was not supported by confocal micrographs taken at this time point. Alternatively, it was possible that, although *C. albicans* could promote GBS attachment to VECs, *C. albicans* may also occlude the receptors that GBS utilises for invasion of VECs. *C. albicans* has been described to interact with epidermal growth factor receptor (Zhu et al., 2012), cadherins such as E-cadherin (Phan et al., 2007), and components of the extracellular matrix

(ECM), including laminin, fibronectin, collagen and vitronectin (Klotz, 1990;Limper and Standing, 1994). Although only one GBS receptor-adhesin pair has been identified (cytokeratin-4 and SRR-1), GBS has also been described to interact with ECM components, including collagen, fibronectin and laminin (Samen et al., 2007;Spellerberg et al., 1999;Beckmann et al., 2002;Banerjee et al., 2011). As such, GBS and *C. albicans* could target similar components of the ECM. However, these interactions have not been explicitly associated with subsequent epithelial internalisation.

For a 'piggy-back' mechanism to occur, candidal hyphae must invade the VECs and so consideration was also given as to whether or not this would occur after a 2 h incubation. Studies have been carried out with oral epithelia which found that *C. albicans* did invade at 2 h, or even as early as 45 min post-inoculation (Phan et al., 2007;Dalle et al., 2010). Nonetheless, invasion dynamics may be tissue-specific, and (Shroff et al., 2017) found that hyphae of *C. albicans* strain 3153A did not invade VECs until 6 h post-infection. Levels of internalised GBS NEM316 and COH1 were significantly higher when *C. albicans* was present compared to monospecies control after 6 h incubation. In line with (Shroff et al., 2017), this supports the hypothesis that *C. albicans* can carry GBS into VECs. Such an effect was not seen, however, with GBS strain 515, which may indicate that 515 preferentially bound VECs over *C. albicans* hyphae. Further experiments would be needed to confirm the dynamics of *C. albicans* and GBS internalisation into VECs. For example, differential antibody-labelling techniques could be used to distinguish between external and internalised cells, although this would be challenging when using 2 microorganisms simultaneously.

Correlating with levels of internalised GBS and *C. albicans*, 6 h assays were also associated with the highest levels of cytotoxicity, as determined by LDH assay. LDH normally exists within the cytoplasm of cells, thus if quantities of LDH above background levels are detected, these are assumed to be from lysed or severely damaged cells. Brightfield and confocal micrographs showed no evidence of significant lysis of the VEC monolayers after 6 h, and these cells were able to support considerable numbers of internalised GBS that would otherwise have been killed by the antibiotics in the surrounding growth medium. Nonetheless, cell rounding was evident, indicating that the VECs were under considerable stress after 6 h, and levels of cytotoxicity were highest in the presence of both microbes rather than with GBS or *C. albicans* alone. This is perhaps unsurprising, as both microbes are known to mediate cytotoxic effects. For example, *C.*

albicans produces candidalysin, which damages the membranes of epithelial cells, while β-H/C expressed by GBS can form pores in the membranes of a variety of host cells (Liu et al., 2004a;Moyes et al., 2016). However, the level of cytotoxicity measured following dual-species infection was greater than the two monospecies cytotoxicity levels combined. This implies that *C. albicans* and GBS may also have synergistic effects on their pathogenic potential. Investigation of the effects on specific virulence determinants and ultimately use of relevant disease models would be needed to explore this in more detail.

There are some potential limitations with the microbial association assays used in this chapter. GBS grow as chains of bacteria, but these may appear as a single colony by viable count, which could lead to an underestimation of the number of bacteria present. In particular, this may have affected GBS strain 2603V/R, which exhibited especially long chains of bacteria when bound to VECs, while strains NEM316, 515 and COH1 appeared to form smaller chains of similar lengths. Nonetheless, overall levels of VEC association for all five GBS strains were in the range of 1-3x10^6 CFU/monolayer. Additionally, microscopy revealed that multiple GBS were often bound to one *C. albicans* hypha, however these bacteria would generate only one colony on agar. Similarly, *C. albicans* can form extensive mats of hyphae across the surface of the VECs. These aggregates can be difficult to dissociate into individual cells, which could again lead to an underestimation of cell number by viable count. Furthermore, *C. albicans* cells in hyphal form will contribute more biomass, yet appear as the same number of colonies by viable count. Nonetheless, there was no evidence that GBS strains modulated candidal hyphae formation and thus the numbers of hyphal filaments should have been similar across the experimental groups in any given assay.

The experiments described in this chapter aimed to determine whether there was an interaction between GBS and *C. albicans*, and whether this might affect the colonisation of VECs by either species. Taken together, the results presented in this chapter describe a synergistic interaction between *C. albicans* and GBS. *C. albicans* is shown to promote GBS association with VECs, and vice versa.

Chapter 4 Mechanistic basis of GBS-*C. albicans* interactions

4.1 Introduction

The data presented in Chapter 3 implied that GBS and *C. albicans* were interacting synergistically to promote colonisation of VECs. It was considered likely that these interactions would be mediated, at least in part, by surface protein interactions. Due to the precedent for antigen I/II family proteins and Als3 in facilitating *Streptococcus-C. albicans* interactions, these adhesins were the primary candidates. Nonetheless, data presented in Chapter 3 also raised the possibility that the GBS-*C. albicans* synergism may be mediated by non-physical means, such as diffusible chemical signals or modulation of microbial growth. Studies to investigate all of these potential mechanisms are the focus of this chapter.

4.1.1 AgI/II family proteins

Antigen I/II (AgI/II) family proteins were first discovered in *Streptococcus mutans* in 1980 (Russell et al., 1980) and are adhesins that protrude from the bacterial cell surface. Their name derives from the fact that these proteins were originally thought to be two separate proteins: antigen I and antigen II; however, antigen II was later found to be the breakdown product of antigen I (Kelly et al., 1989). Since this discovery, AgI/II proteins have been found in practically all oral streptococcal species, including *S. gordonii*, *S. oralis* and *S. intermedius* (Ma et al., 1991;Demuth et al., 1996). More recently, AgI/II proteins have been characterised in Group A *Streptococcus* (GAS) and GBS (Zhang et al., 2006;Chuzeville et al., 2015;Sitkiewicz et al., 2011).

4.1.1.1 AgI/II structure

The primary sequence of AgI/II family proteins can be divided into 7 distinct domains: an N-terminal leader peptide, an N-terminal domain, an alanine-rich domain (A domain), a variable region, a proline-rich domain (P domain), a C-terminal domain and a cell wall anchor containing an LPxTG motif (Figure 4-1). The N-terminal leader domain directs the protein to the Sec secretion system, by which it is transported across the membrane until the sorting signal (the LPxTG motif) is reached. Upon translocation across the cell

membrane, the protein folds in half with the variable domain presented at the 'tip', while α-helices of the A domains and the polyproline II-helices of the P domains interact, enabling these domains to coil around one another and form the 'stalk' of the protein (Rego et al., 2016b). The N-terminal domain stabilises the protein structure by interacting with the C-terminal domain which is anchored to the cell wall (Figure 4-1). The variable region is thought to enable specificity of binding (Brady et al., 2010).

The gene encoding GAS AgI/II protein, AspA, is present on an integrative and conjugative element named region of difference 2 (RD2), and it has been proposed that this was acquired by GAS via horizontal gene transfer from GBS (Sitkiewicz et al., 2011;Brochet et al., 2008;Zhang et al., 2006). Four homologues of GBS AgI/II proteins have been described, designated BspA-D. Bsp proteins can be split into two groups based on homology, BspA/B and BspC/D. BspA and BspB share over 90% similarity, while BspD is essentially identical to BspC except for the absence of the leader peptide for targeting to the Sec translocation machinery (Rego et al., 2016b). When compared against BspC/D proteins, BspA/B proteins have two extra sequences; an additional 51 amino acids in the A domain and an additional 25 amino acids in the P domain (Rego et al., 2016b). Bsp proteins share high sequence homology (39-42% amino acid identity) with the AgI/II proteins of GAS (Chuzeville et al., 2015;Rego et al., 2016b).

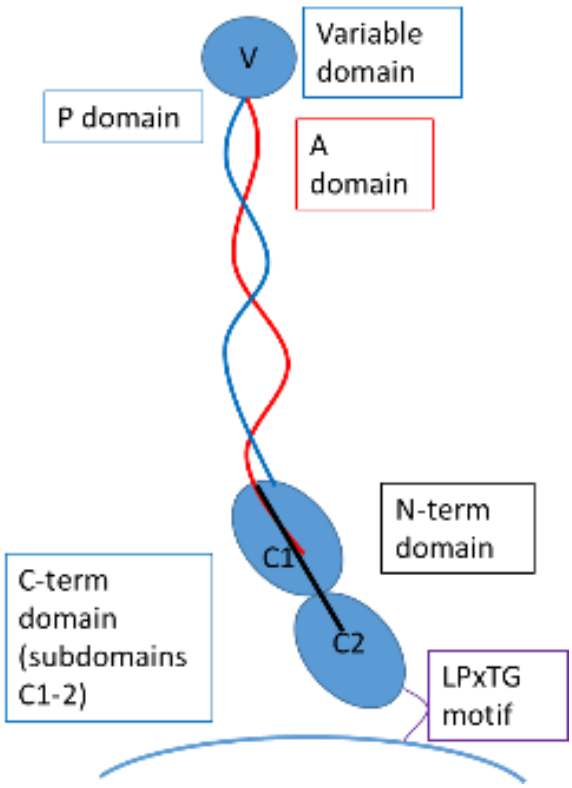

Figure 4-1 Structure of the AgI/II family proteins of GBS, which have been named Bsp proteins.

The V region is presented at the tip of the protein, while the A domain (red) and the P domain (blue) coil around one another to form a supporting 'stalk' structure. The N-terminal domain (black) stabilises the protein through interactions with the C-terminal domains, which anchor the protein to the cell surface via the LPxTG motif. Adapted from (Rego et al., 2016b).

4.1.1.2 AgI/II functions

AgI/II proteins have been most widely researched in oral streptococci. Streptococcal AgI/II family proteins are multifunctional and have been found to aid streptococcal colonisation, biofilm development and interactions with other microorganisms (Franklin et al., 2013;Hall et al., 2014;Xu et al., 2014a). For example, *S. gordonii* AgI/II proteins SspA/B have been shown to aid in association with and invasion of HEp-2 cells (Nobbs et al., 2007). AgI/II family proteins are also able to bind to a wide variety of host molecules, including laminin, collagen and salivary proteins such as glycoprotein-340 (gp-340) (Brady et al., 2010). Gp-340 is produced at mucosal surfaces and functions to aggregate microbes for expulsion from the body when secreted in liquid-phase, but can also act as a receptor for microbial attachment when in surface-bound phase (Loimaranta et al., 2005). For example, *S. mutans* AgI/II protein SpaP binding to gp-340 plays a major role in facilitating attachment to the salivary pellicle and enabling colonisation of the oral cavity (Brady et al., 2010). The AgI/II proteins of both GAS and GBS have also been found to

bind gp-340 (Zhang et al., 2006;Rego et al., 2016b). Alongside binding host molecules, AgI/II proteins have been shown to be involved in promoting colonisation of host tissues by binding to other microorganisms, such as *Actinomyces oris*, *Porphyromonas gingivalis*, and *C. albicans* (Jakubovics et al., 2005;Lamont et al., 2002;Silverman et al., 2010). Interactions between *C. albicans* and oral streptococci have been described as synergistic, with microorganisms cooperating in order to persist for longer within the oral cavity (Shirtliff et al., 2009). AgI/II family proteins have been shown to contribute to this synergism.

4.1.2 Als3 proteins

Als3 is a cell surface protein expressed by *C. albicans* that is a member of the agglutinin-like sequence (Als) gene family (Hoyer et al., 2008). One study found that >70% of *C. albicans* isolated from vaginal swabs taken from women with vaginal thrush express Als3 (Roudbarmohammadi et al., 2016). Thus, it is likely that Als3 is expressed in the vaginal tract. Coleman et al. (2009) published fluorescence micrographs of *C. albicans* cells that had been probed with an anti-Als3 antibody, with the result that only the *C. albicans* hyphae were labelled (Coleman et al., 2009). This is particularly relevant given the hyphal tropism GBS was observed to exhibit.

4.1.2.1 *ALS3* gene structure

ALS genes all share a similar three-domain structure (see Figure 4-2), with a conserved 5' domain, a central domain comprising of a variable number of tandemly repeated copies of an 108 base pair motif, and a 3' domain that differs in length and sequence across the genes (Hoyer et al., 1998b). There is variation in the number of tandem repeats in each *ALS* gene between *C. albicans* strains and alleles, however the most common number of repeats for *ALS3* in *C. albicans* SC5314 are 9 or 12 (Hoyer et al., 2008). The difference in the number of tandem repeats was found to correlate with function, with the larger protein demonstrating greater adhesive abilities than the smaller protein when cultured with vascular endothelial or pharyngeal epithelial cells (Oh et al., 2005). However, it was found that *C. albicans* possesses one large *ALS3* allele and one short allele (Oh et al., 2005).

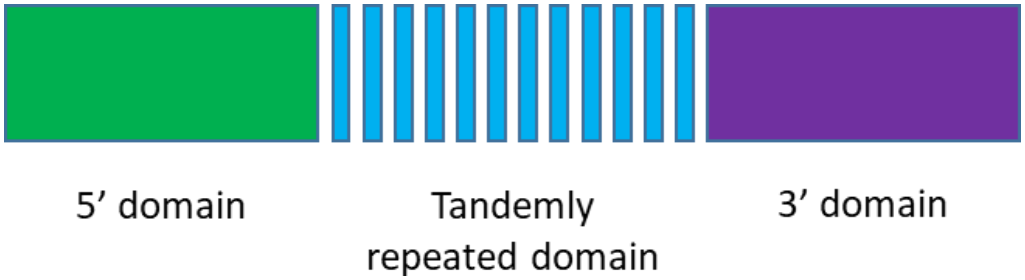

Figure 4-2 *ALS* gene structure.

The 5’ domain is conserved in length and sequence across the *ALS* gene family. However, the tandemly repeated domain and 3’ domain are variable in length. In *ALS3*, the 3’ domain is relatively short (879 bp). Adapted from (Hoyer et al., 1998b).

4.1.2.2 Als3 function

Monoclonal antibodies against Als3 were able to reduce adhesion by *C. albicans* to FaDu oral epithelial cells, and Als3 was found to be required for induced endocytosis by two oral epithelial cell lines and human umbilical vein endothelial cells (Phan et al., 2007). Furthermore, a *C. albicans als3/als3* double mutant was significantly reduced in oral epithelial adhesion and damage (Zhao et al., 2004a). This highlights the role of Als3 as an important *C. albicans* adhesin. Additionally, Als3 was found to specifically bind to N-cadherin and E-cadherin on endothelial and oral epithelial cells, respectively (Phan et al., 2007).

Alongside interactions with host substrata, Als3 has been implicated in the interactions between *C. albicans* and a number of bacterial species. These include *S. aureus*, which has been shown to specifically bind Als3 (Peters et al., 2012). Via this interaction, *S. aureus* is carried into host epithelial cells as *C. albicans* hyphae invade, and this has been described as the main mechanism for *S. aureus* host tissue invasion (Schlecht et al., 2015a). *Rothia dentocariosa* and *C. albicans* are the most common microorganisms identified in mixed species biofilms on failed voice implants, and it was found that *R. dentocariosa* also targets Als3 on *C. albicans* hyphae (Uppuluri et al., 2017). *C. albicans* had been described to interact with the AgI/II proteins of *S. gordonii* (Bamford et al., 2009). When investigating this interaction further, (Silverman et al., 2010) found that a *als3/als3* double mutant was ablated in coassociation with *S. gordonii*, whether in biofilms or suspension. Heterologous protein expression of Als3 on *Saccharomyces cerevisiae* and SspB on *L. lactis* showed that the *C. albicans-S. gordonii* interaction was facilitated by specific protein binding between Als3 and SspB (Silverman et al., 2010).

4.1.3 Non-physical interactions

Most sites of the body are colonised by communities consisting of multiple species of microorganisms, which are sustained by inter-species interactions. While many of these interactions involve direct receptor-ligand binding between microbial surface molecules, this is not the only mechanism by which microbes can influence one another. A number of microbial species have been shown to interact with one another indirectly. Rather than specific binding of microbial cell surface adhesins, these microbes may interact through excretion of small diffusible signals, modulating host responses or by altering the dynamics of the host environment in a way which is beneficial to other organisms.

Microorganisms can produce metabolites which may benefit other microorganisms. This has been described for the synergistic interaction between *Treponema denticola* and *Porphyromonas gingivalis*. Biofilm growth of *T. denticola* is promoted by *P. gingivalis* production of isobutyric acid, while growth of *P. gingivalis* is likewise promoted by *T. denticola* production of succinic acid and chemotrypsin-like proteinase (Grenier, 1992;Cogoni et al., 2012). Additionally, modulating the environment may promote growth of other microorganisms. For example, *Fusobacterium nucleatum* has been shown to enhance *P. gingivalis* biofilm formation by reducing the amount of oxygen, enabling anaerobic growth (Bradshaw et al., 1998). Small, diffusible molecules may also be produced which facilitate growth of other microorganisms. For example, interspecies biofilm interactions between *S. gordonii* and *Veillonella atypica* are facilitated by *V. atypica* inducing expression of the *amyB* amylase gene by *S. gordonii* (Egland et al., 2004). Upregulation of amylase when in the presence of digestible starch leads to larger quantities of glucose, which can be fermented to produce lactic acid, promoting *V. atypica* (Egland et al., 2004). Furthermore, autoinducer-2 produced by *S. gordonii* has been found to induce formation of hyphae in *C. albicans* by suppressing the quorum sensing molecule farnesol (Bamford et al., 2009). In mixed species biofilms between *S. mutans* and *C. albicans*, *sigX*-induced competence was triggered in *S. mutans*. This was also triggered by the supernatant from mixed, but not single-species, biofilms (Sztajer et al., 2014). Although *C. albicans* typically exists in its yeast form at low pH, when co-incubated with oral streptococci, *C. albicans* can form hyphae at pH <4.5. Furthermore, streptococci producing H_2O_2 trigger oxidative stress and thus induce formation of hyphae by *C. albicans* (Jenkinson et al., 1990;Nasution et al., 2008).

4.2 Results

4.2.1 Expression of AgI/II family proteins

(Rego et al., 2016b) showed that GBS carries genes encoding AgI/II family proteins. However, the conditions under which these proteins are expressed, or the levels of expression were not determined. The first step of these studies was therefore to test for expression of AgI/II family proteins on the surface of GBS. This included obtaining antibodies against BspA and BspC, which were generated in rabbits using full-length recombinant proteins purified from *E. coli* as the antigens (see Chapter 2 for details).

To validate these antibodies, aliquots from overnight broth cultures of GBS strain NEM316 (Figure 4-3, upper rows) or 515 (Figure 4-3, lower rows) and successive 1:2 dilutions were dotted onto nitrocellulose membranes. Membranes were exposed to either anti-Bsp purified antibody or pre-immune sera from the rabbits used to generate either BspA or BspC antibody, as indicated (Figure 4-3). As anticipated, NEM316 and 515 reacted with BspA or BspC sera respectively in a dose-dependent manner (Figure 4-3). Importantly, no reactivity was seen with the preimmune sera, confirming the specificity of antibody binding. Cross-reactivity was also observed, as NEM316 reacted with anti-BspC antibody, and 515 with anti-BspA antibody, despite these strains not expressing the target antigen. This is likely due to the high degree of sequence similarity between the two proteins (Rego et al., 2016b).

Figure 4-3 Dot immunoblots to indicate Bsp expression by GBS.

Aliquots (2 µL) from overnight THY broth cultures of strains NEM316 (upper rows) or 515 (lower rows) were transferred to nitrocellulose membranes, alongside five successive 1:2 dilutions (from right to left). Membranes were then probed with either purified anti-BspA or anti-BspC antibody (top panels) or corresponding rabbit pre-immune sera (bottom panels), followed by a secondary antibody conjugated to horseradish peroxidase, and developed by chemiluminescence.

In an attempt to better visualise surface expression of BspA and BspC, and to explore the effects of environmental parameters or growth phase on expression levels, GBS strains NEM316 and 515 were grown at different temperatures in an overnight broth culture, or to either early or late exponential growth phase. Cells were fixed in PFA and stained with anti-BspA or -BspC antibodies, as appropriate. Cells were then probed with a secondary antibody conjugated to Alexafluor-555 and visualised by fluorescence microscopy (Figure 4-4). NEM316 was found to have a greater level of reactivity compared to strain 515 under each of the conditions tested (Figure 4-4, left panels), which may be due to cross-reactivity of the antibody with BspB alongside BspA. However, a similar binding profile was seen for both strains, in that only a small proportion (approximately 10%) of the cell population was brightly labelled by the antibodies. For some cells the antibody labelling appeared to cover the entire surface (Figure 4-4, white arrows), while other cells exhibited a punctate binding pattern (Figure 4-4, blue arrows). Labelling of bacteria grown to early exponential phase (OD_{600} 0.25) was brighter than for those grown to late exponential phase (OD_{600} 0.9), but of the two growth phases, early exponential phase exhibited a higher proportion of punctate-labelled cells. Incubation temperature also affected expression of the Bsp proteins (Figure 4-4), with a larger proportion of cells fully labelled by the antibodies at 30 and 34 °C compared to cells grown at 37 °C. Taken together, these data confirmed surface expression of Bsp proteins by GBS strains NEM316 and 515, but implied that overall expression levels may be strain-dependent and affected by growth phase and temperature.

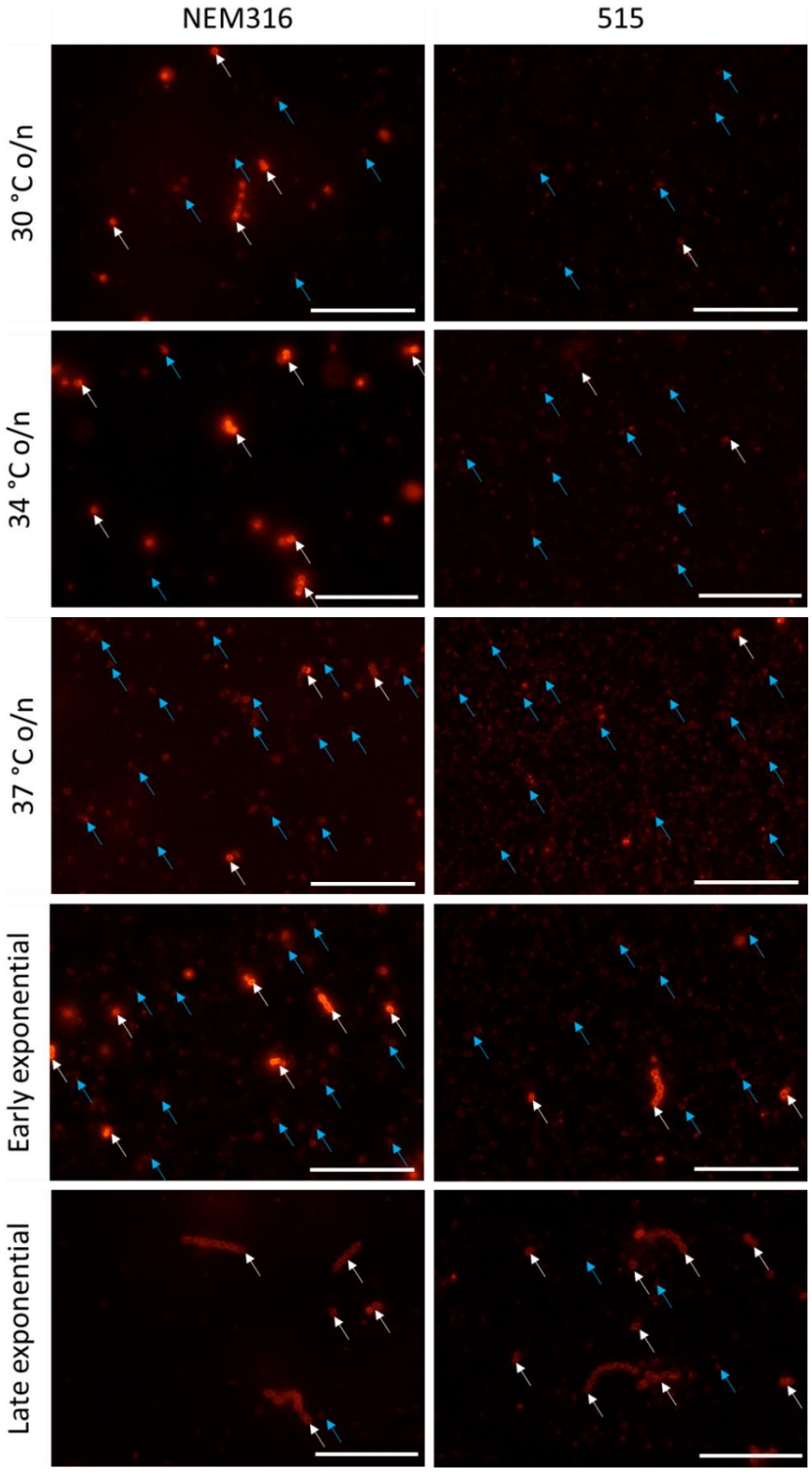

Figure 4-4 Expression of BspA and BspC under differing growth conditions.

GBS strains NEM316 (left panels) and 515 (right panels) were grown overnight in THY broth at 30 °C, 34 °C or 37 °C, 5% CO_2 as indicated. For investigations on the effect of growth phase on AgI/II family protein expression, bacteria were grown at 37 °C, 5% CO_2 in an overnight broth culture before cells were adjusted to an OD_{600} of 0.1. Bacteria were then grown to either early (OD_{600} ~0.25) or late (OD_{600} ~0.8) exponential growth phase. Bacteria were probed with either α-BspA (NEM316) or α-BspC (515) antibodies, followed by an Alexafluor-555-conjugated secondary antibody. White arrows indicate GBS which is entirely labelled by anti-Bsp antibody, while blue arrows indicate GBS which is partially labelled. Scale bars, 20 µm.

4.2.2 Direct Bsp interactions with VECs

AgI/II family homologues from other streptococci have been implicated in colonisation and invasion of host tissues (Nobbs et al., 2007). As such, initial studies explored the capacity for Bsp proteins to influence VEC colonisation, using *bsp* gene knockout and complemented mutant strains. These strains were made by (Rego et al., 2016b). NEM316 has one copy of the *bspA* gene and three copies of the *bspB* gene, while 515 and COH1 have only one copy of the *bspC* gene (Rego et al., 2016b). These latter two strains were therefore selected for mutagenesis.

WT, Δ*bspC* and Δ*bspC+bspC* strains of 515 and COH1 were tested for association and invasion of VECs (Figure 4-5). There was a significant and almost identical reduction in association and invasion levels of 39% and 41%, respectively, for 515Δ*bspC* relative to WT (Figure 4-5A, B). Likewise, COH1Δ*bspC* was significantly impaired in association and invasion of VECs (Figure 4-5C, D) compared to WT, but the reduction was more extensive than seen for strain 515 at 95% and 94% for association and invasion, respectively. For both GBS strains, complementation with *bspC* restored association and invasion to WT levels (Figure 4-5).

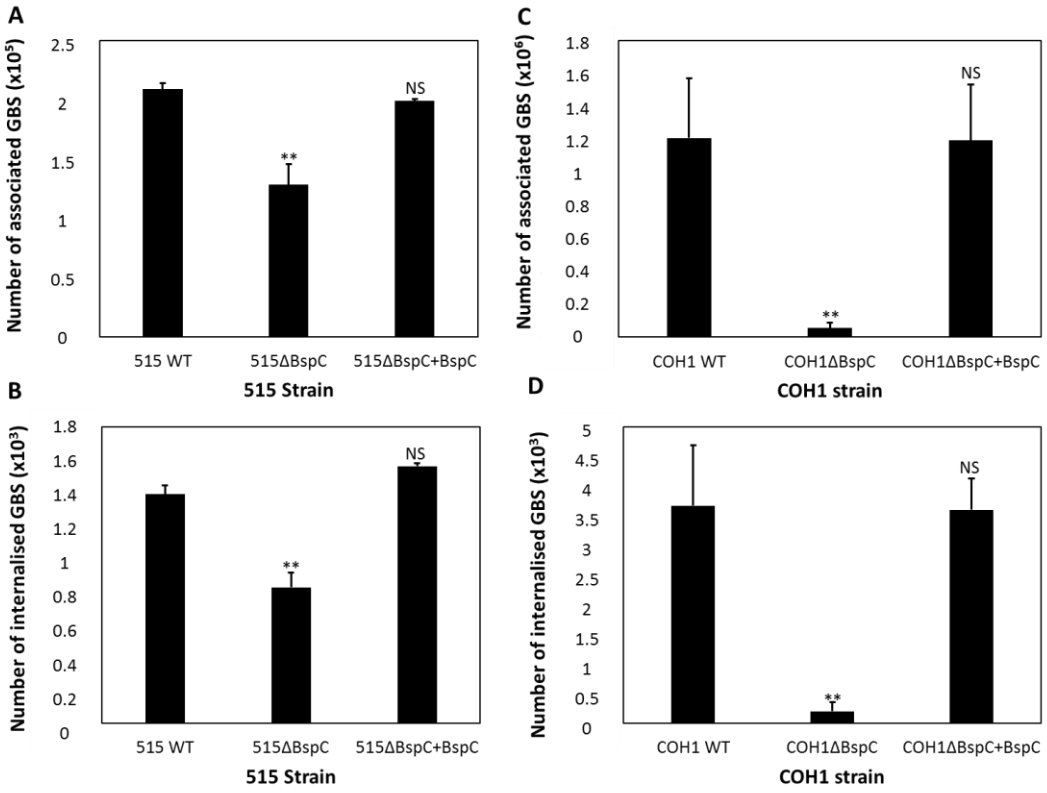

Figure 4-5 Effects of BspC expression on GBS interactions with VECs.

VEC monolayers were incubated with (A,B) 515 WT or (C,D) COH1 WT and corresponding BspC knockout (ΔBspC) or complemented (ΔBspC+BspC) strains (MOI 25) for 1 h. Monolayers were either (A,C) lysed (for association) or (B,D) incubated with media containing 200 μg/mL gentamicin and 10 μg/mL penicillin for 2 h (for invasion) prior to lysis. Numbers of associated/internalised GBS were then enumerated by serial dilution onto THY agar. Data are presented as mean ± SD, ** indicates $P<0.01$ when compared to WT, NS indicates $P>0.01$ when compared to WT, as determined by unpaired Student's t-test with Bonferroni correction; n=3.

To further verify the contribution of BspC to mediating GBS interactions with VECs, and to investigate the potential role of BspA also, antibody inhibition studies were performed. For these assays, GBS strains 515 or NEM316 were incubated with either pre-immune sera or anti-Bsp antibody for 30 min prior to incubation with VECs (Figure 4-6). For both GBS strains, compared to pre-incubation with preimmune sera, there was a significant decrease of 50-60% in the number of GBS able to associate with VECs when pre-incubated with anti-Bsp antibody (Figure 4-6). Taken together, these data indicate a role for BspA and BspC in mediating GBS interactions with VECs.

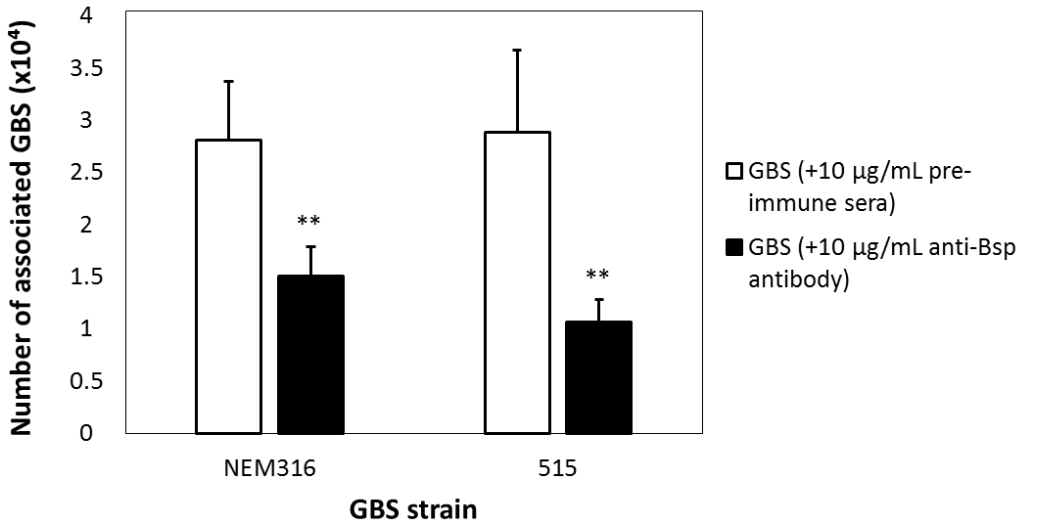

Figure 4-6 Effects of anti-Bsp antibodies on GBS interactions with VECs.

GBS (MOI 2.5) was pre-incubated for 30 min with 10 µg/mL anti-BspA (NEM316) or -BspC (515) antibody, or 10 µg/mL pre-immune sera as control. Bacteria were then added to wells containing VECs and incubated for 1 h. Monolayers were lysed and numbers of GBS were enumerated by serial dilution onto THY agar. Data are presented as mean ± SD, ** indicates P<0.01 as determined by unpaired Student's t-test with Bonferroni correction; n=3.

4.2.3 Role of Bsp proteins in coassociation with *C. albicans*

The AgI/II family protein SspB of *S. gordonii* has been found to specifically associate with *C. albicans* (Silverman et al., 2010). To explore if a similar function could be ascribed to the Bsp proteins of GBS and if this influenced the coassociation seen with VECs, VEC association assays were performed with strains 515 WT, 515Δ*bspC* and 515Δ*bspC+bspC*, in the presence or absence of *C. albicans* (Figure 4-7). As was observed in Figure 4-5, there was a significant reduction in the number of 515Δ*bspC* able to associate with VECs when compared to 515 WT (Figure 4-7A). However, the 86% reduction was greater than seen previously and more similar that shown for strain COH1 in Figure 4-5. Association levels for all three 515 strains were significantly promoted by 3.6-fold for WT, 6.4-fold for Δ*bspC* and 3.4-fold for Δ*bspC+bspC* in the presence of *C. albicans* (Figure 4-7A). However, absolute numbers of 515Δ*bspC* recovered remained significantly lower than those of WT or 515Δ*bspC+bspC* either without (6-7-fold lower) or with (3-4-fold lower) *C. albicans*. Numbers of recovered *C. albicans* cells were significantly higher in the presence of each of the three 515 strains (Figure 4-7B). Of potential note, however, was that while *C. albicans* numbers were promoted by 3.3-fold and 3.2-fold by 515 WT and 515Δ*bspC+bspC* respectively, levels were only 1.9-fold elevated in the presence of 515Δ*bspC.* Thus, these data imply that BspC plays a role in the coassociation seen

between *C. albicans* and GBS, but that it is not the sole GBS adhesin responsible for these effects.

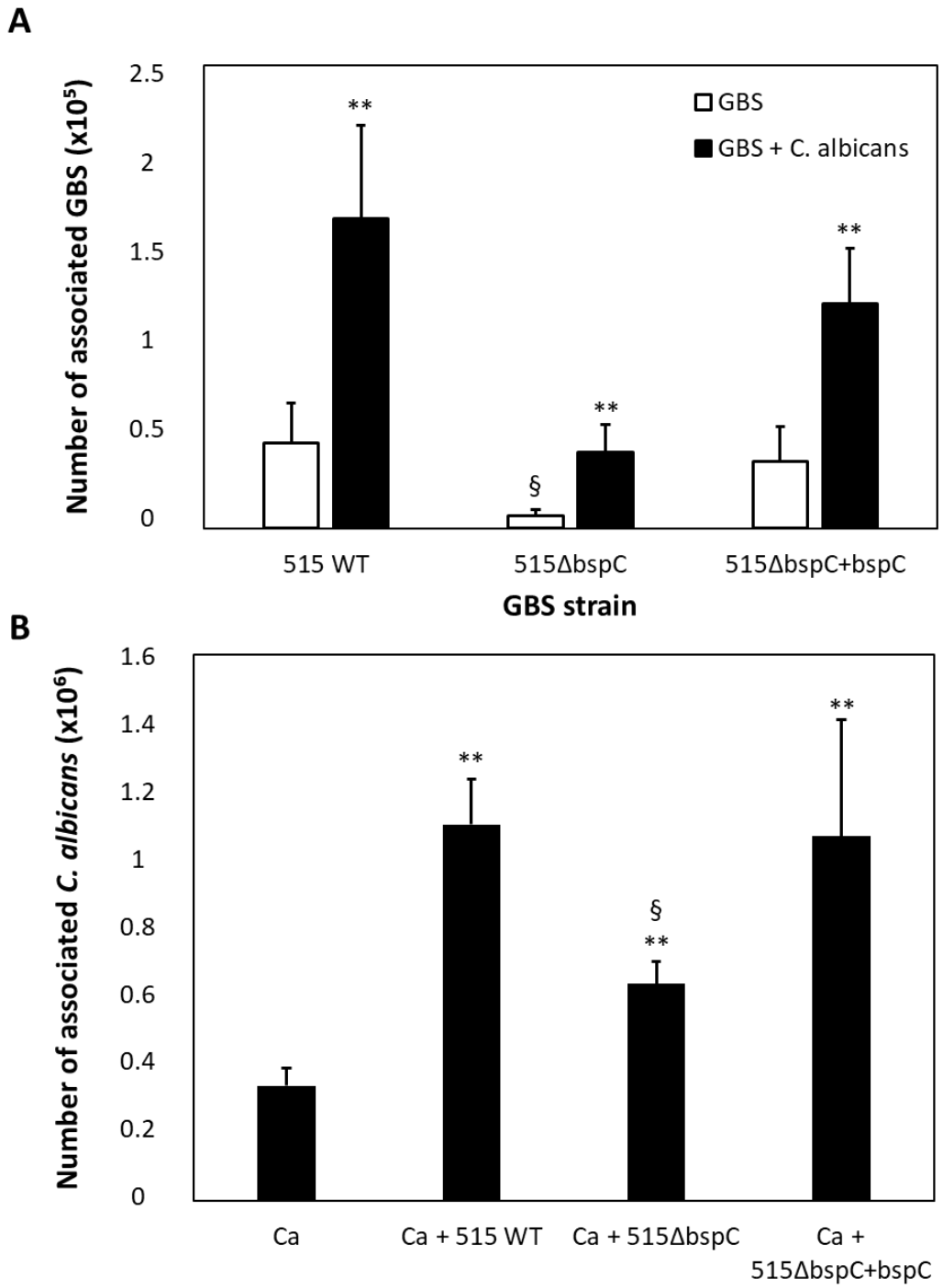

Figure 4-7 Effects of BspC expression on interactions of GBS and *C. albicans* with VECs.

VEC monolayers were incubated with *C. albicans* (MOI 2.5) for 2 h, or with GBS 515 WT, 515Δ*bspC* and 515Δ*bspC*+*bspC* (MOI 2.5) for 1 h. Alternatively, monolayers were incubated with *C. albicans* for 1 h followed by incubation with GBS for a further 1 h. Monolayers were lysed and (A) GBS or (B) *C. albicans* were enumerated by serial dilution onto THY agar supplemented with 50 µg/mL nystatin or SAB agar supplemented with 5 µg/mL erythromycin, respectively. Data are presented as mean ± SD, ** indicates P<0.01 relative to monospecies control, § indicates P<0.01 relative to 515 WT (A) or *C. albicans*+515 WT (B) as determined by one-way ANOVA with Tukey post-test; n=4.

To further investigate the role of AgI/II family proteins in mediating GBS interactions with VECs and with *C. albicans,* while avoiding potential issues with functional redundancy across other GBS proteins, Gram positive surrogate host *Lactococcus lactis* was used to express BspA or BspC. The *bspA* or *bspC* gene was carried on plasmid pMSP7517 under the control of nisin-inducible promoter P*nisA*, enabling levels of heterologous protein expression to be regulated by addition of nisin to the growth medium (Rego et al., 2016b). These surrogate expression strains were then tested in VEC association assays in the presence or absence of *C. albicans*. There was a modest yet significant increase in numbers of *L. lactis* expressing BspA or BspC recovered from VECs relative to the empty vector control strain (Figure 4-8). For all three strains of *L. lactis*, numbers of recovered cells were significantly elevated in the presence of *C. albicans*. However, for the empty vector control strain this increase was only 1.4-fold, while the BspA-expressing strain was promoted by 2.8-fold, and the BspC-expressing strain by 4-fold (Figure 4-8). This provided further evidence that the AgI/II family proteins facilitate the interactions of GBS with VECs and *C. albicans*.

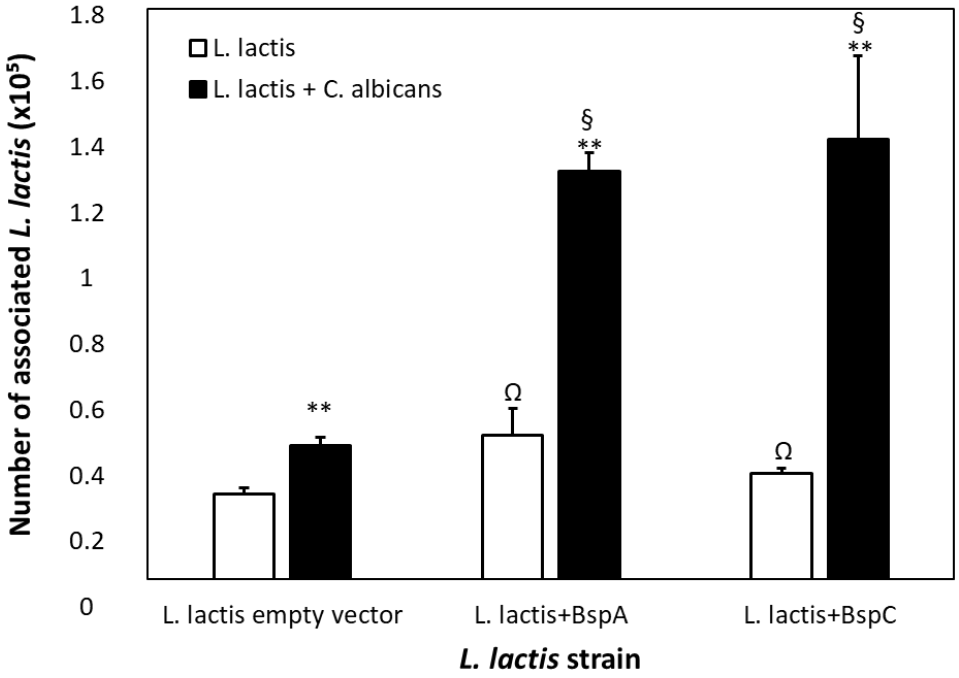

Figure 4-8 Effects of *C. albicans* on the association of *L. lactis* Bsp surrogate expression strains with VECs.

VEC monolayers were incubated with suspensions of *L. lactis* pMSP vector control, pMSP.BspA or pMSP.BspC (MOI 2.5) for 1 h (white bars), or with *C. albicans* (MOI 2.5) for 1 h followed by addition of *L. lactis* suspensions for a further 1 h (black bars). Monolayers were then lysed and numbers of associated *L. lactis* were enumerated by serial dilution onto GM17 agar supplemented with 50 µg/mL nystatin. Data are presented as mean ± SD, ** indicates significance relative to monospecies controls; Ω indicates significance relative to pMSP empty vector control; § indicates significance relative to pMSP empty vector control in the presence of *C. albicans*. Significance indicates P<0.01, as determined by one-way ANOVA with Tukey post-test; n = 4.

The *L. lactis* surrogate expression strains were also utilised to explore the role of Bsp proteins in VEC invasion, and any potential influence of *C. albicans* on this. These studies were carried out in a similar way to the invasion experiments detailed for Figure 4-5 with the exception that, due to the non-invasive nature of *L. lactis*, the multiplicity of infection (MOI) had to be increased to 200 before the number of colonies recovered was quantifiable. As observed with GBS in Chapter 3, there was no significant difference between the numbers of internalised *L. lactis* in the monospecies controls when compared against *L. lactis* incubated with *C. albicans* (Figure 4-9). However, for *L. lactis* alone, there was a significant increase in the numbers of internalised bacteria when expressing BspA or BspC relative to the empty vector control. This amounted to a 4.4-fold increase for BspA, and a 2.5-fold increase for BspC. There was no statistically significant difference between *L. lactis* invasion levels upon expression of BspA compared to BspC. However, Figure 4-9 suggests that BspA may facilitate invasion with greater efficiency than BspC, as on average invasion levels were 2-fold higher for *L. lactis* expressing BspA than BspC.

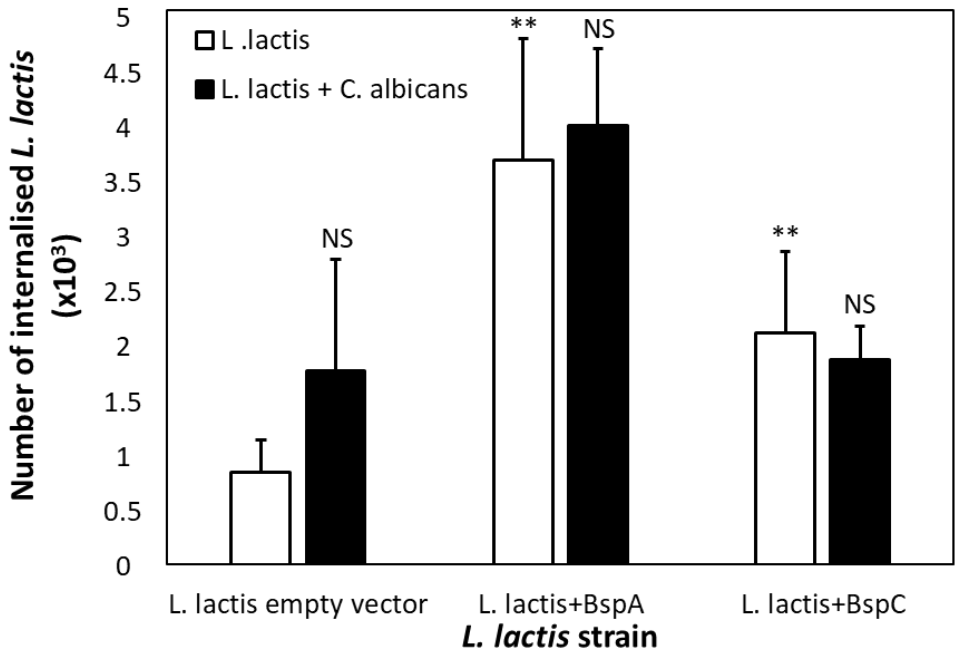

Figure 4-9 Effects of Bsp expression or *C. albicans* on invasion of VECs by *L. lactis*.

VEC monolayers were incubated for 1 h with *C. albicans* (MOI 2.5), before *L. lactis* pMSP vector only control, pMSP.BspA or pMSP. BspC were added (MOI 200) and incubated for a further 1 h. Monolayers were then incubated with media containing 200 µg/mL gentamicin and 10 µg/mL penicillin to kill any extracellular bacteria, before lysis and enumeration of internalised *L. lactis* by serial dilution onto GM17 agar plates supplemented with 50 µg/mL nystatin. Data are presented as mean ± SD, ** indicates P<0.01 when compared to pMSP vector control, NS indicates P>0.01 when compared to monospecies controls, as determined by one-way ANOVA with Tukey post-test; n=4.

4.2.4 Role of Als3 in coaggregation with GBS

As discussed, there is evidence to suggest that *C. albicans* mediates interactions with other microbial species via adhesin Als3 (Peters et al., 2012). To investigate whether or not Als3 was involved in the interactions with GBS, a homozygous mutant strain of *C. albicans* was used in which *ALS3* was deleted (designated *C. albicans*Δ*als3*), alongside the corresponding complemented strain (designated *C. albicans*Δ*als3+als3*) (Zhao et al., 2004a). Initially, the role of Als3 in mediating planktonic (coaggregation) interactions of *C. albicans* with GBS was investigated. *C. albicans* was grown in YNBPTG for 2 h, before addition of GBS and incubation for a further 1 h. Cells were differentially labelled with fluorescent dyes and then visualised by fluorescence microscopy (Figure 4-10A). Levels of coaggregation were semi-quantitatively determined by scoring hyphae based on the number of interacting GBS (Figure 4-10B). GBS strains NEM316 and 515 exhibited strong interactions with *C. albicans* WT*,* with 40-50% of the hyphae achieving a score of >20 bound GBS. By contrast, these strains were not able to coaggregate strongly with *C. albicans*Δ*als3*, for which either 0 or 1-5 bound GBS was scored for the majority of hyphae. The number of bacteria interacting with *C. albicans* Δ*als3+als3* was similar to WT levels, suggesting that complementation of Als3 restored the WT coaggregation phenotype. Strain COH1 rarely interacted with any of the three *C. albicans* strains. However, numbers of hyphae devoid of bound COH1 were higher for *C. albicans*Δ*als3* compared to WT (Figure 4-10). Taken together, these data suggested that *C. albicans* adhesin Als3 plays a major role in mediating coaggregation with permissible GBS partner strains.

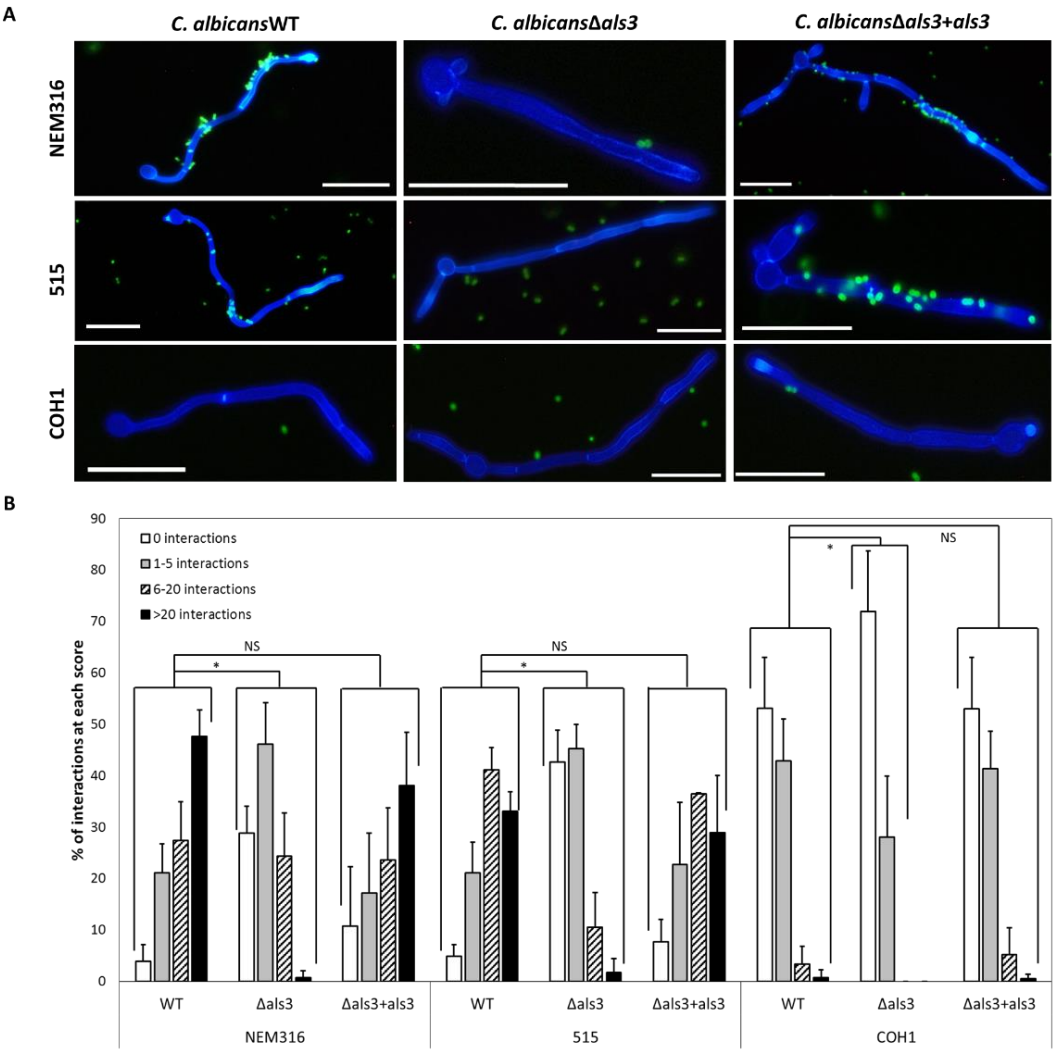

Figure 4-10 Role of Als3 in coaggregation of *C. albicans* with GBS.

(A) Representative fluorescence micrographs of planktonic interactions between *C. albicans* WT (left panel), Δ*als3* (middle panel) or Δ*als3+als3* (right panel) and GBS strains NEM316 (top panels), 515 (middle panels) or COH1 (bottom panels). *C. albicans* was grown in YNBPTG for 2 h at 37 °C, 220 rpm before addition of GBS and incubation for a further 1 h. GBS was labelled with FITC (green), while *C. albicans* was labelled with Calcofluor White (blue). Scale bars, 20 µm. (B) Semi-quantitation of numbers of *C. albicans* hyphae with 0 interacting GBS, 1-5, 6-20 or >20 interacting GBS. Data are presented as mean ± SD, * P<0.05; NS P>0.05, as determined by linear regression analysis of datasets; n = 4.

While the data presented in Figure 4-10 highlighted Als3 in facilitating *C. albicans*-GBS coaggregation, it was not possible to ascertain from these data which adhesin(s) on the surface of GBS were involved. Previous studies had shown that AgI/II family protein SspB can specifically interact with Als3 (Silverman et al., 2010). To determine if such functions extended to the Bsp adhesins of GBS, coaggregation assays were repeated with the *L. lactis* heterologous expression strains and the *C. albicans* Als3 strains (Figure 4-11). The *L. lactis* pMSP vector only control strain coaggregated weakly with *C. albicans*,

regardless of strain, and there was no significant difference in the semi-quantitatively scored interactions with any of the three *C. albicans* strains (Figure 4-11B). *L. lactis* expressing BspA or BspC tended to bind along the length of the *C. albicans* WT or *C. albicans*Δ*als3+als3* hyphae (Figure 4-11A). However, neither strain bound *C. albicans*Δ*als3*, and semi-quantitative analysis revealed that *L. lactis* expressing BspA or BspC coggregated at significantly lower levels with *C. albicans*Δ*als3* than with WT (Figure 4-11B). There were no significant differences in coaggregation levels between *C. albicans* WT and *C. albicans*Δ*als3+als3*, with the majority of hyphae exhibiting >20 bound *L. lactis* expressing BspA or BspC. Thus, Als3 was shown to be critical for coaggregation between *C. albicans* and the Agl/II-expressing *L. lactis* strains. These data raised the possibility that there may be a specific Als3-Bsp interaction.

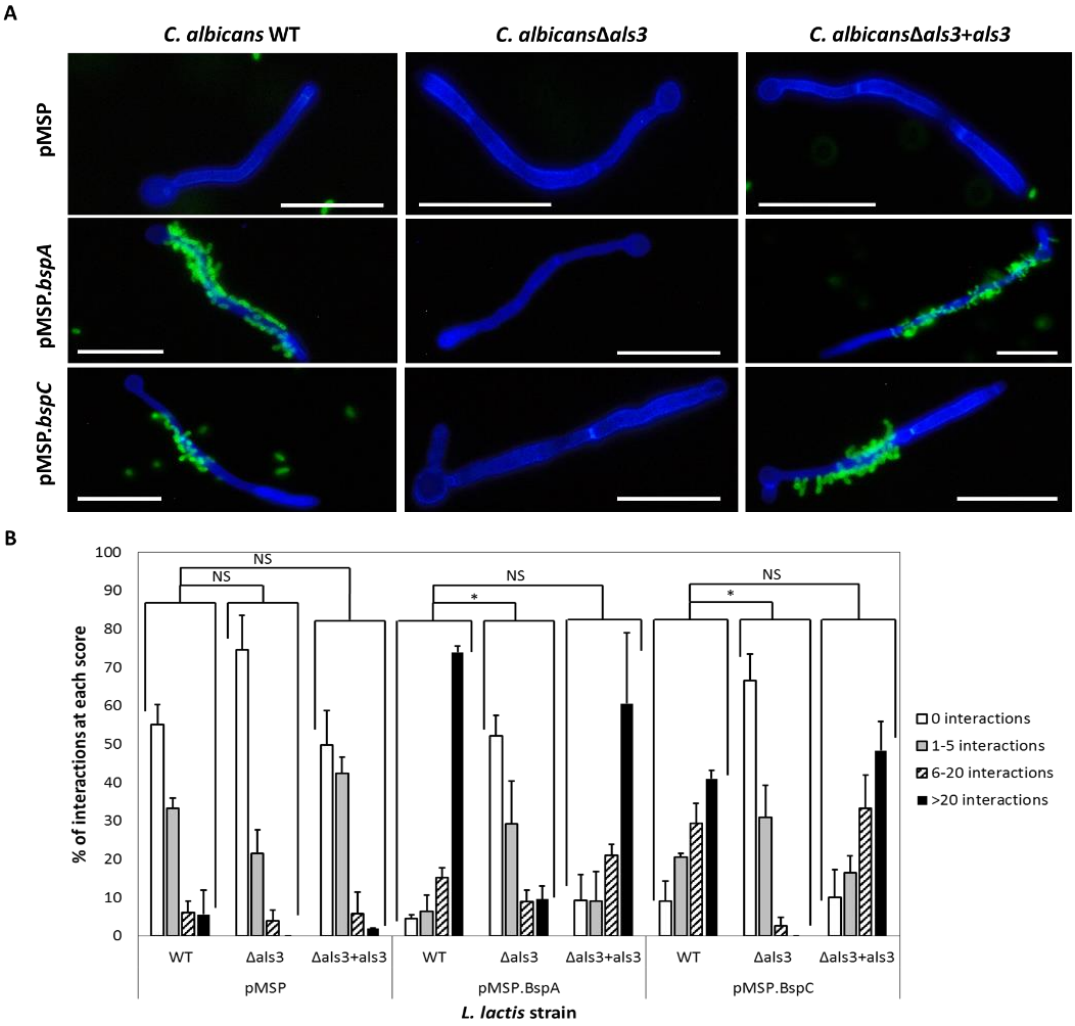

Figure 4-11 Role of Als3 in coaggregation of *C. albicans* with *L. lactis* Bsp-expressing strains.

(A) Representative fluorescence micrographs of planktonic interactions between *C. albicans* WT (left panel), Δ*als3* (middle panel) or Δ*als3+als3* (right panel) and *L. lactis* strains pMSP vector control (top panels), pMSP.BspA (middle panels) or pMSP.BspC (bottom panels). *C. albicans* was grown in YNBPTG for 2 h at 37 °C, 220 rpm before addition of *L. lactis* and incubation for a further 1 h. GBS was labelled with FITC (green), while *C. albicans* was labelled with Calcofluor White (blue). Scale bars, 20 µm. (B) Semi-quantitation of numbers of *C. albicans* hyphae with 0 interacting *L. lactis*, 1-5, 6-20 or >20 interacting *L. lactis*. Data are presented as mean ± SD, * P<0.05; NS P>0.05, as determined by linear regression analysis of datasets; n = 4.

4.2.5 Role of Als3 in coassociation with GBS

Having demonstrated a role for *C. albicans* Als3 in mediating coaggregation with GBS, the next step was to determine if this interaction also influenced the coassociation seen between these microbes on VECs. *C. albicans* WT, *C. albicans*Δ*als3* and *C. albicans*Δ*als3+als3* were incubated with VECs in the presence or absence of GBS (Figure 4-12). As before, there was a significant increase in the numbers of GBS recovered from VECs in the presence of *C. albicans* WT, and comparable effects were seen with *C. albicans*Δ*als3+*als3. NEM316 was promoted by 2.3-fold, 515 by 6.1-fold, and COH1 by 5-

fold (Figure 4-12A). Reciprocal effects were also seen for *C. albicans*, with numbers of associated *C. albicans* WT or *C. albicans*Δ*als3+als3* being enhanced by approximately 3-fold in the presence of each GBS strain (Figure 4-12B). By contrast, there were no significant differences in GBS association levels when incubated with *C. albicans*Δ*als3* compared to the monospecies control (Figure 4-12A), and the numbers of recovered *C. albicans*Δ*als3* in the presence of GBS were comparable to those of the monospecies control (Figure 4-12B). These data strongly implied that the synergistic promotion of GBS-*C. albicans* association with VECs was dependent on Als3.

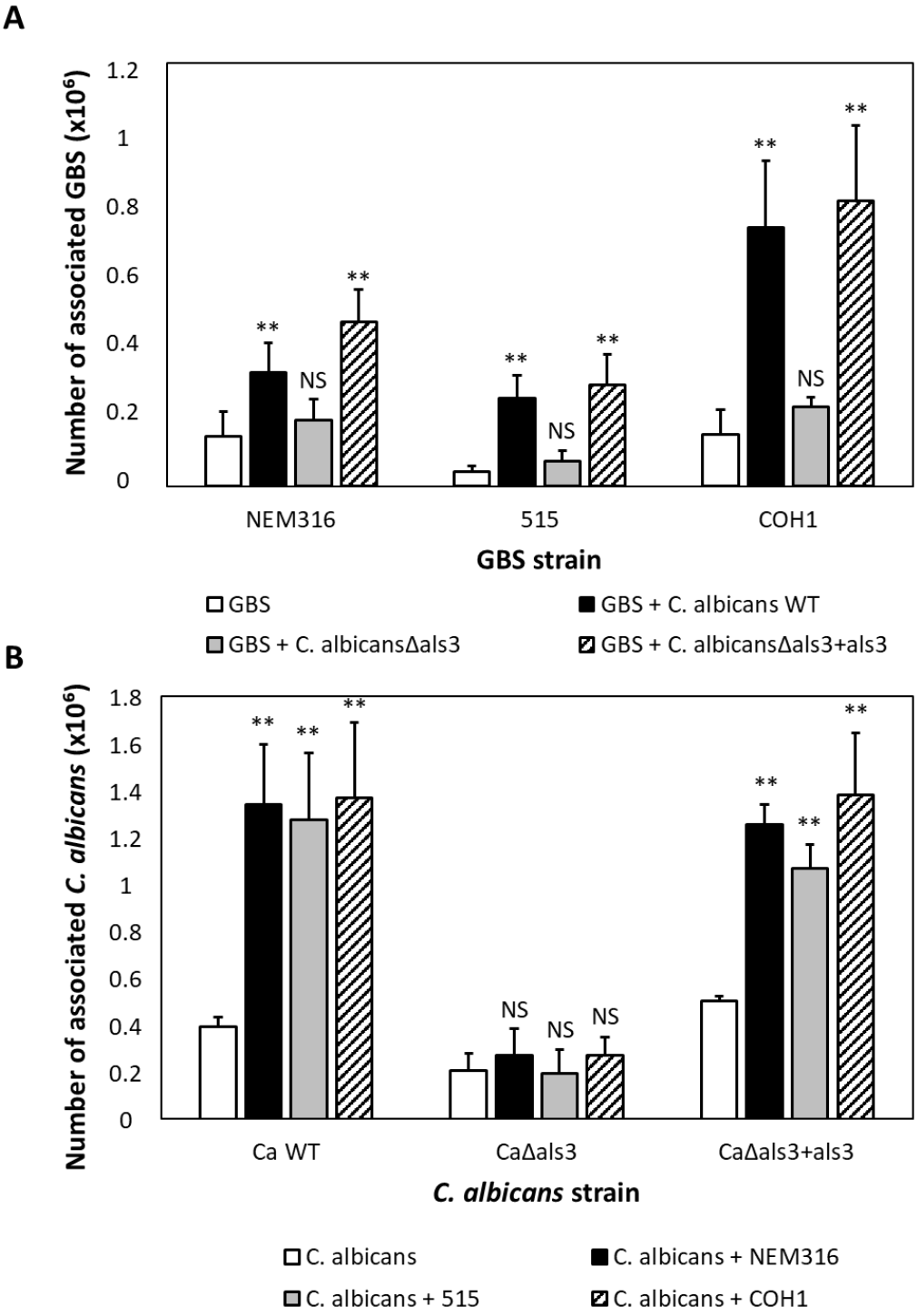

Figure 4-12 Role of Als3 in coassociation of *C. albicans* and GBS with VECs.

(A) VEC monolayers were incubated with GBS suspensions for 1 h (white bars) or with *C. albicans* WT (black bars), Δ*als3* (grey bars) or Δ*als3+als3* (striped bars) for 1 h followed by GBS for a further 1 h. Monolayers were lysed and then numbers of associated GBS were enumerated by serial dilution onto THY agar supplemented with 50 µg/mL nystatin. (B) VEC monolayers were incubated with *C. albicans* WT, Δ*als3* or Δ*als3+als3* for 1 h followed by GBS strains NEM316 (black bars), 515 (grey bars) or COH1 (striped bars) for a further 1 h. Monolayers were lysed and numbers of associated *C. albicans* were enumerated by serial dilution onto SAB agar supplemented with 5 µg/mL erythromycin. Data are presented as mean ± SD; ** P<0.01, NS P>0.01, as determined by one-way ANOVA with Tukey post-test; n = 4.

4.2.6 Direct Bsp-Als3 interaction

These studies had demonstrated a role for Bsp adhesins of GBS and Als3 of *C. albicans* in mediating coaggregation between these two microbes and in their coassociation on VECs. However, while strongly implicated, direct binding between these adhesins had not been shown. To try and resolve this, coaggregation assays were performed using *L. lactis* Bsp-expressing strains together with a *Saccharomyces cerevisiae* Als3 heterologous expression strain (Figure 4-13). The *L. lactis* pMSP vector control strain did not interact with *S. cerevisiae* expressing Als3 (Figure 4-13, left panel). However, *L. lactis* expressing BspA (Figure 4-13, middle panel) or BspC (Figure 4-13, right panel) coaggregated with Als3-expressing *S. cerevisiae*. These micrographs provided supporting evidence for a direct Als3-Bsp interaction.

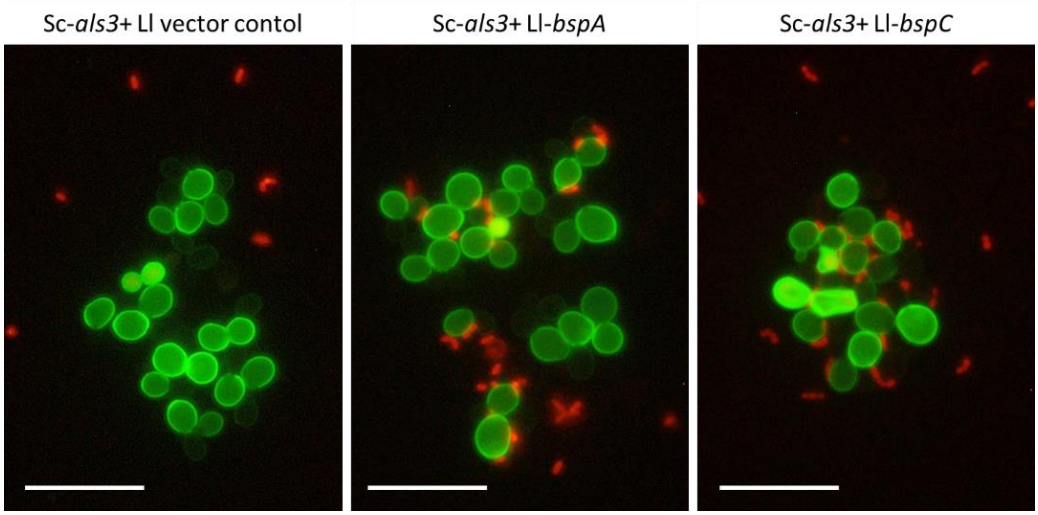

Figure 4-13 Fluorescence micrographs of planktonic interactions between *S. cerevisiae* Als3 and *L. lactis* Bsp surrogate expression strains.

S. cerevisiae (Als3+) was grown in YNBPTG for 3 h at 30 °C, 220 rpm before addition of *L. lactis* pMSP control (left), *L. lactis* pMSP.BspA (middle) or *L. lactis* pMSP.BspC (right) and incubation for a further 1 h. *L. lactis* was labelled with TRITC (red), while *S. cerevisiae* was labelled with FITC (green). Scale bars, 20 µm.

4.2.7 Non-physical interactions

4.2.7.1 Effects on microbial growth

In Chapter 3 it was found that the promotion of GBS association with VECs may not be entirely dependent on direct binding with *C. albicans*. Confocal micrographs revealed that, although there were many bacteria bound to *C. albicans* hyphae (indicating a direct, physical interaction between the two organisms), there was also a visible increase in the number of adherent bacteria to the epithelium in areas where *C. albicans*

was not present. To investigate whether this could have been due to an increase in the overall number of microorganisms present resulting from enhanced growth/replication, GBS and *C. albicans* were grown together planktonically in conditions to mirror an association assay and the CFU/mL determined by viable count and compared to monocultures (Figure 4-14). Numbers of neither GBS (Figure 4-14A) nor of *C. albicans* (Figure 4-14B) displayed any significant difference between the dual-species and monospecies cultures, suggesting that neither species significantly affected growth of the other.

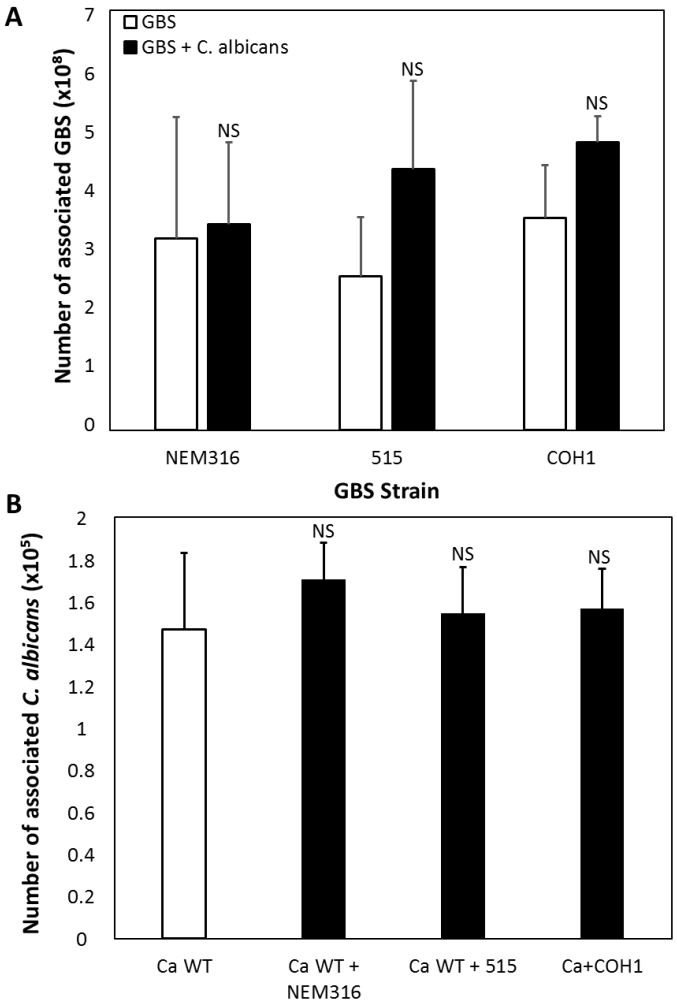

Figure 4-14 Effects of mono- or dual-species incubation on numbers of *C. albicans* or GBS in suspension culture.

K-SFM broth cultures were inoculated with *C. albicans* (MOI 2.5) at 37 °C, 220 rpm for 1 h before addition of GBS and incubation for a further 1 h (black bars). Alternatively, broth cultures were inoculated with *C. albicans* or GBS alone and incubated for 2 h or 1 h respectively (white bars). GBS CFU/mL were then determined by viable count onto THY agar supplemented with 50 µg/mL nystatin (A), while *C. albicans* CFU/mL were determined by viable count onto SAB agar supplemented with 5 µg/mL erythromycin (B). Data are presented as mean ± SD, NS indicates P>0.05 compared to the monospecies control, as determined by unpaired Student's t-test; n = 3.

4.2.7.2 Role of diffusible signals

It is known that alongside physical interactions, chemical communication exists in polymicrobial communities. Thus, it was hypothesised that small diffusible molecules may be produced by GBS or *C. albicans* that could affect their interactions with VECs. To explore this possibility, VEC association assays were performed as before with the modification that GBS was physically separated from *C. albicans* using transwell plates. *C. albicans* were pre-incubated with VECs before GBS were added in the top compartment of transwell baskets (Figure 4-15). In theory, if chemical signals were produced by GBS that promoted *C. albicans* interactions with VECs, these would be able to pass through the small pores in the transwell membrane and mediate their effects. However, there were no significant differences in the numbers of *C. albicans* cells recovered from VECs in the presence of GBS under these conditions compared to *C. albicans* alone (Figure 4-15).

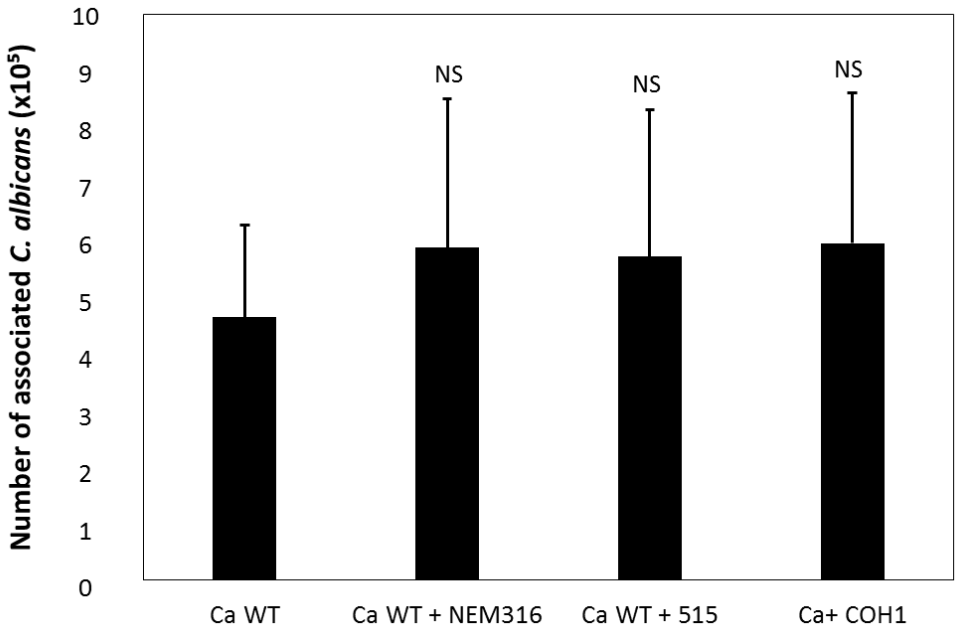

Figure 4-15 Association of *C. albicans* with VECs when exposed to GBS in a contact-independent manner.

C. albicans was grown on VEC monolayers for 1 h, before GBS suspensions or K-SFM alone were placed into transwell baskets suspended above. After a further 1 h incubation, *C. albicans* was enumerated by serial dilution onto SAB agar. Data are presented as mean ± SD, NS indicates P>0.01, as determined by unpaired Student's t-test with Bonferroni correction; n=3.

Another possibility was that stimulatory signals may only be produced when microbes are in contact with the VECs. To investigate this hypothesis, *C. albicans* was grown with

VECs in conditioned media from GBS grown either planktonically or with VECs, and vice versa (Figure 4-16). Conditioned media were sterilised through a 0.4 µm pore filter before inoculation with GBS or *C. albicans* and incubation with VEC monolayers. There were no significant differences in the numbers of GBS associated with VECs in conditioned media from *C. albicans* grown planktonically (Figure 4-16A, striped bars) or incubated with VECs (Figure 4-16A, black bars) when compared against the blank medium control (Figure 4-16A, white bars). Likewise, there were no significant differences in the numbers of *C. albicans* associated with VECs when incubated in GBS planktonic or VEC conditioned media relative to blank medium control (Figure 4-16B).

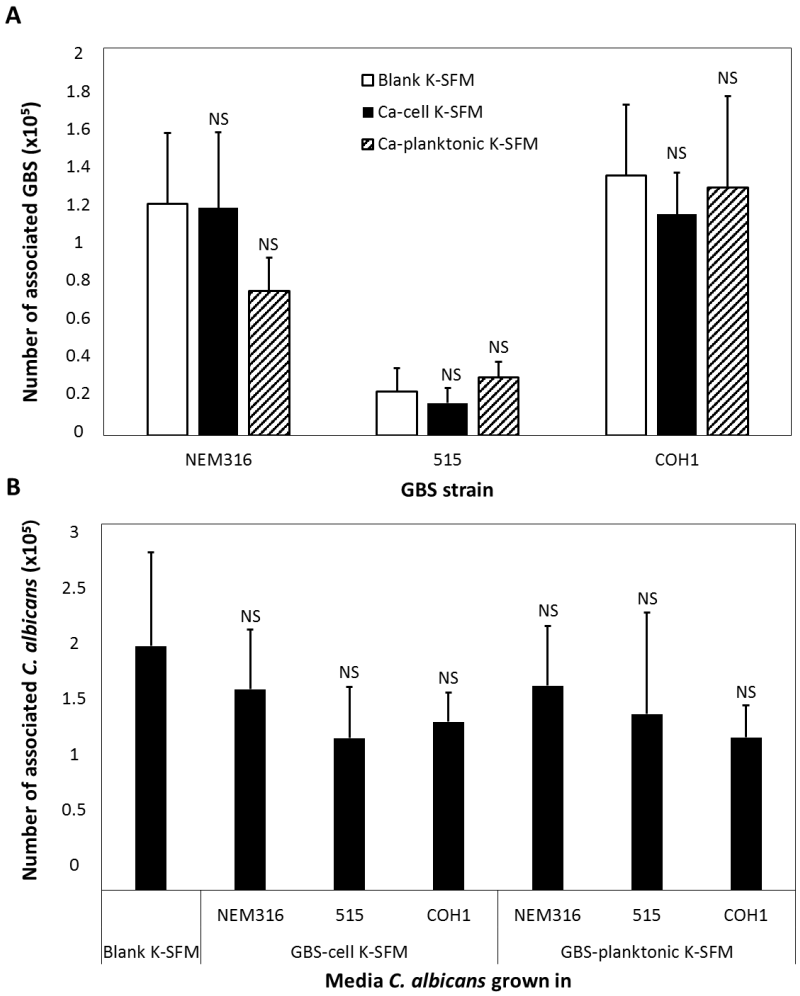

Figure 4-16 Effects of conditioned media on microbial association with VECs.

A) VEC monolayers were incubated with *C. albicans* (MOI 2.5) for 1 h. Alternatively, *C. albicans* was incubated planktonically at 220 rpm for 1 h. Suspensions were collected and filter sterilised. GBS was diluted into either fresh K-SFM or *C. albicans* conditioned media and incubated with fresh VECs (MOI 2.5) for 1 h. Monolayers were lysed and GBS was enumerated by serial dilution onto THY agar; n=5. B) VEC monolayers were incubated with GBS strains NEM316, 515 or COH1 (MOI 2.5) for 1 h. Alternatively, GBS were grown statically in suspension for 1 h. Suspensions were collected and filter sterilised. *C. albicans* was diluted into either fresh K-SFM or GBS conditioned media, and incubated with fresh VECs (MOI 2.5) for 1 h. Monolayers were lysed and *C. albicans* was enumerated by serial dilution onto SAB agar. Data are presented as mean ± SD; n=3; NS indicates that P>0.05, as determined by one-way ANOVA with Tukey post-test.

4.2.7.3 Effects of VEC fixation on GBS-*C. albicans* coassociation

While chemical signals did not seem to play a significant role in GBS-*C. albicans* coassociation with VECs, one final mechanism to explore was if *C. albicans* may interact with VECs to make them more permissive to GBS. Such a process would require an active response from VECs and so the effects of VEC fixation on this coassociation were investigated. VEC monolayers were fixed with paraformaldehyde prior to incubation with GBS and/or *C. albicans*. Fixation of VECs did reduce the overall levels of association for GBS strains NEM316 and 515 (Figure 4-17A, white bars). However, association levels were significantly promoted by ~20-fold in the presence of *C. albicans* for both GBS strains (Figure 4-17A, black bars) relative to GBS alone. These effects of *C. albicans* were greater than seen in previous assays, but this likely reflects the fact that much lower levels of GBS association were seen for the monospecies controls. Fixation did not significantly impact the capacity for *C. albicans* to bind VECs (Figure 4-17B). In the presence of GBS NEM316 or 515, numbers of recovered *C. albicans* cells were promoted by 2.4-fold or 2.9-fold respectively compared to *C. albicans* alone (Figure 4-17B).

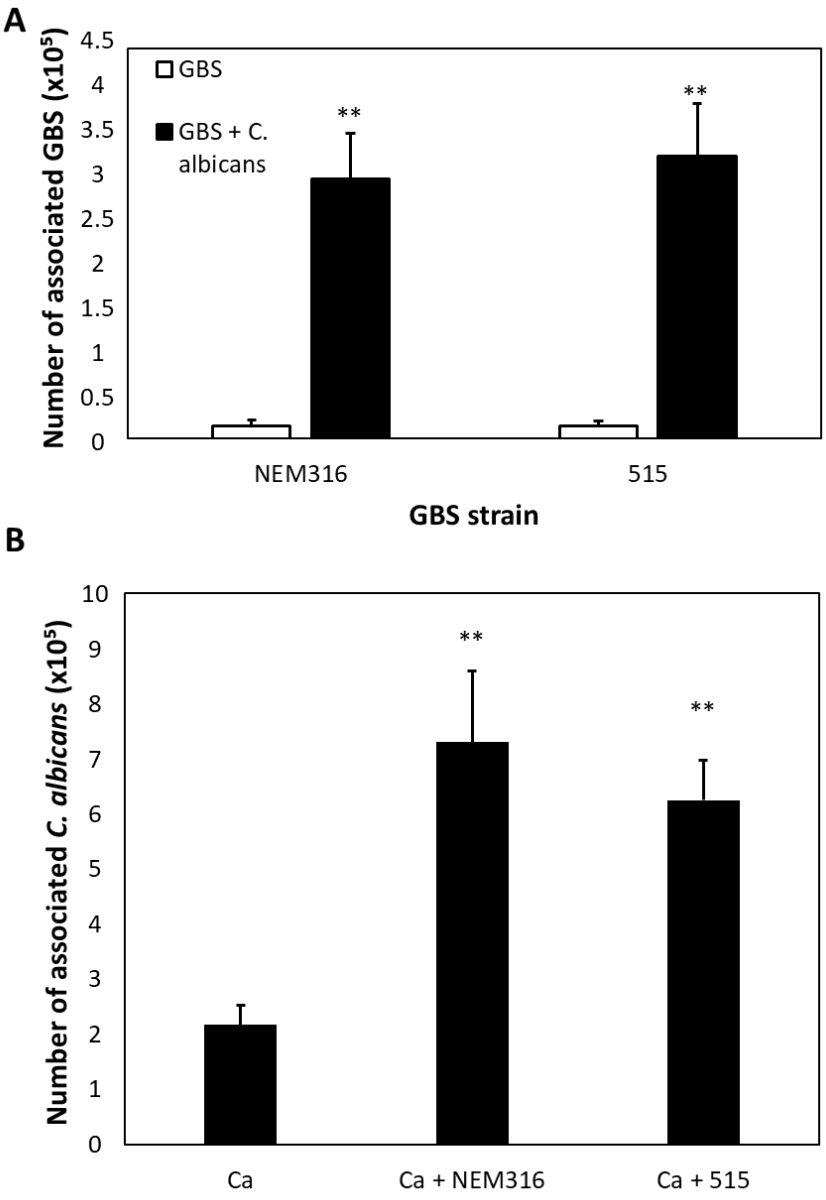

Figure 4-17 Effects of VEC fixation on GBS-*C. albicans* interactions.

VEC monolayers were fixed with 2% paraformaldehyde overnight, before A) incubation with GBS suspensions (MOI 2.5) for 1 h (white bars) or with *C. albicans* (MOI 2.5) for 2 h, or with *C. albicans* for 1 h followed by GBS for a further 1 h (black bars). Monolayers were then lysed and GBS CFU/mL determined by viable count onto THY agar supplemented with 50 µg/mL nystatin, or (B) *C. albicans* CFU/mL were determined by viable count onto SAB agar supplemented with 5 µg/mL erythromycin. Data are presented as mean ± SD, ** indicates $P<0.05$ relative to the monospecies control, as calculated by unpaired Students t-test; n=3.

Taken together, these data imply that neither GBS nor *C. albicans* produce diffusible signals that significantly influence the association of these microbes with VECs. Furthermore, no active response from VECs is required to facilitate these synergistic

effects. Rather, it seems likely that the direct physical interaction of these microorganisms is the critical mechanism that underpins their coassociation with VECs.

4.3 Discussion

The data presented in this chapter explored the molecular basis for GBS-*C. albicans* interactions. Investigations into non-physical interactions found neither chemical signals nor active response by VECs to play a significant part in the coassociation between GBS and *C. albicans*. Thus, the implication was that these interactions were predominantly driven via physical interactions between the two microbes, for which AgI/II family (Bsp) proteins and Als3 were found to be the principal mediators.

Antibodies raised against BspA and BspC were used to confirm surface expression by GBS strains NEM316 and 515 and these exhibited cross-reactivity between the two strains. NEM316 possesses one copy of *bspA* and three copies of *bspB*, while 515 possesses only one copy of *bspC*. Aside from the additional sequences in the A and P domains of the BspA/B homologues when compared to BspC/D homologues, there is a high level of sequence identity between these proteins (Table 4-1). This likely explains why the antibodies appeared to label both strains comparably.

Table 4-1 Domain similarity across Bsp proteins.

% amino acid identity given for each of the AgI/II domains when compared against BspA. Adapted from (Rego et al., 2016b).

Bsp protein	Leader peptide	N domain	A domain	V region	P domain	C domain	Cell wall anchor
BspB	87%	97%	97%	100%	88%	90%	64%
BspC	90%	97%	96%	97%	96%	83%	82%
BspD	N/A	97%	97%	100%	88%	90%	64%

Expression of the AgI/II proteins was monitored under a variety of conditions, including different temperatures and growth phases. Two populations of labelled GBS were observed: completely labelled cells and partially labelled cells. When bacteria were grown to early exponential growth phase (at 37 °C), both populations of cells were

observed, while at late exponential phase, cells were mostly completely labelled. This implies that Bsp expression may be constitutive but that levels of surface expression are optimal as the cells enter stationary phase. There are examples of such expression variation within this adhesin family, as for *S. gordonii* the *sspA* promoter was found to increase activity throughout growth, while the *sspB* promoter induced most activity at late exponential phase (El-Sabaeny et al., 2000). This aspect of Bsp expression could be further investigated in the future if reporter strains were generated using the *bsp* promoter sequences, thereby allowing transcription of the *bsp* genes to be monitored throughout the growth cycle. It was also noted that fluorescence levels with the anti-Bsp antibodies were brighter in early rather than late exponential phase. This may indicate impaired antibody access as the GBS cell matures and produces increasing levels of capsular polysaccharide, which could be explored further by including capsular staining in these experiments. Only around ~10% of the bacteria viewed under brightfield microscopy were clearly fluorescent. However, more information would be needed about the sensitivity of the anti-Bsp antibodies to confirm whether the remaining GBS cells were expressing Bsp at lower levels.

Completely labelled GBS cells were more prevalent at 30 and 34 °C, whereas partially labelled cells were most common at 37 °C. This implies that Bsp expression may be temperature-responsive. Smoot et al. (2001) described a number of GAS proteins which were regulated in response to temperature (29 ^{o}C vs. 37 ^{o}C), with 28 proteins containing secretion signals differentially expressed according to temperature (Smoot et al., 2001). Furthermore, expression of the *S. gordonii* AgI/II proteins SspA and SspB has been found to vary in response to temperature, as well as pH and osmolarity (El-Sabaeny et al., 2000). At 34 °C, the SspB promoter induced similar expression as at 37 °C, while the SspA promoter was decreased in activity (El-Sabaeny et al., 2000). Temperature-related regulation of proteins can be advantageous to bacteria by conferring the ability to colonise different host environments. Although GBS is predominantly found in the gastrointestinal and genitourinary tracts, GBS is also capable of causing a range of diseases in non-pregnant adults. These normally present as non-specific bacteraemia or infections of the skin and soft tissue, although in rare cases GBS may cause endocarditis or meningitis (Fujita et al., 2015;Scully et al., 1987b;Sambola et al., 2002). To survive, GBS must therefore be able to adapt and grow under the different conditions, including temperature, found in these multiple niches. It is interesting to note that pH also affected expression of *S. gordonii* SspA/B proteins, with a neutral pH favoured by both

promoters (El-Sabaeny et al., 2000). As the vaginal environment is more acidic than the oral cavity, it could be hypothesised that expression of Bsp proteins may occur over a larger pH range; however, this needs further investigation.

It is widely documented that AgI/II family proteins are important adhesins of streptococci, particularly those that colonise the oral cavity (Brady et al., 2010). Rego et al. (2016) showed that BspA expression by *L. lactis* conferred an increased capacity to bind VECs (Rego et al., 2016b). Here, knockout and complemented strains of GBS 515 and COH1 have been used to demonstrate a role for BspC expression in VEC binding and internalisation. There was a significant reduction in numbers of Δ*bspC* strains associated with or internalised within VECs compared to WT, and complementation restored WT phenotype. Furthermore, anti-Bsp antibodies impaired the ability of GBS NEM316 and 515 to interact with VECs by 50%, while *L. lactis* strains expressing BspA or BspC were enhanced in both VEC association and invasion compared to the *L. lactis* vector control strain. These data provide strong evidence that BspA and BspC, and thus possibly all Bsp family proteins, may facilitate vaginal colonisation by GBS through direct interactions with the vaginal mucosa. Nonetheless, *bsp* knockout strains were not ablated in association with VECs, highlighting the common strategy utilised by several bacterial species of adhesin redundancy, whereby bacteria have multiple proteins that carry out the same function to maximise survival (Kline et al., 2009). Bsp proteins may therefore play a role in GBS-GU tract interactions but are not the only GBS proteins involved. There is precedent for AgI/II family proteins binding directly to epithelial cell receptors or to ECM proteins. For example, *S. gordonii* SspA/B can adhere to integrin receptor $\alpha_5\beta_1$ and collagen type I (Heddle et al., 2003;Nobbs et al., 2007), while SpaP of *S. mutans* and Pas of *S. intermedius* have both been found to bind fibronectin (Petersen et al., 2002). Identifying the VEC receptor(s) targeted by GBS Bsp proteins will be the focus of future studies.

Copy numbers of *bsp* genes differ across the GBS strains tested here and it was hypothesised that these differences could affect the capacity of GBS strains to associate with VECs. Bsp antibody labelling, as detected by immunofluorescence, appeared to confirm that NEM316 expresses greater levels of AgI/II proteins on its surface than 515. In turn, GBS NEM316 interacted with VECs in higher numbers than GBS 515. However, in the previous chapter, COH1 was shown to associate with and invade into VECs at similar levels to NEM316 and yet COH1 only carries a single *bspC* gene. This could reflect the

role of other proteins in mediating GBS association with VECs. Alternatively, the different Bsp proteins may exhibit varying binding affinities. Indeed, the *L. lactis* strain expressing BspA interacted with VECs in higher numbers than the *L. lactis* strain expressing BspC. These possibilities may not be mutually exclusive.

The *C. albicans* cell surface adhesin Als3 was identified as a protein that is critical for the interaction between *C. albicans* and GBS. Als3 is hypha-specific (Coleman et al., 2009), which correlates with the tropism GBS was observed to exhibit for hyphae. There was a significant reduction in interactions with *C. albicans* hyphae when all three strains of GBS were incubated with the *C. albicans* Δ*als3* strain relative to WT, while interactions with *C. albicans* Δ*als3+als3* were comparable to WT. Likewise GBS could promote association of *C. albicans* WT and *C. albicans* Δ*als3+als3* with VECs but not of *C. albicans* Δ*als3*. *C. albicans* Δ*als3* was not impaired in formation of hyphae and associated with VECs similar to WT *C. albicans*. Thus, it was explicitly loss of Als3 expression on candidal hyphae that impaired interactions with GBS. There was even a significant reduction of GBS COH1 binding to *C. albicans* Δ*als3* hyphae compared to WT, which is perhaps surprising given the rarity of COH1-*C. albicans* interactions in suspension. This suggests a difference in the protein profile of COH1 cells in VEC association assays when compared to planktonic suspension. However, this would have to be confirmed, for example, with proteomic analyses.

Heterologous expression of BspA or BspC on *L. lactis* conferred the capacity to coaggregate with *C. albicans* expressing Als3 but not *C. albicans* Δ*als3*. Furthermore, these *L. lactis* strains could coaggregate with *Saccharomyces cerevisiae* expressing Als3. These data strongly suggest that Bsp proteins directly target Als3 on *C. albicans* hyphae, as has been found for the *S. gordonii* AgI/II protein SspB (Bamford et al., 2009;O'Sullivan et al., 2000;Jenkinson et al., 1990). Bsp proteins share only 6-22% overall protein identity with SspA/B (Chuzeville et al., 2015) and so it is unclear if common binding domains exist in BspA/C and SspB for engaging Als3. Hoyer et al. (2014) found that *S. gordonii* was significantly less able to bind *C. albicans* Als3 with mutations in the peptide binding cavity of the N-terminus of Als3 (Hoyer et al., 2014), which had been shown to interact with the C-terminus of ECM protein ligands (Lin et al., 2014). However, as SspB is covalently linked to the peptidoglycan of the streptococcal cell wall (Nobbs et al., 2009), the logistics of such a binding mechanism for *S. gordonii* were less clear. To address this, (Hoyer et al., 2014) proposed that portions of the SspB C-terminus were

stably linked to the bacterial cell wall, yet others are free to interact with Als3 following selective proteolytic cleavage by either *C. albicans*, bacterial or even host proteases. A major difference between SspB and Bsp proteins is that while SspB has the typical three C-terminal domains (C1, C2 and C3) (Brady et al., 2010), Bsp proteins of GBS only possess two C-terminal domains, which are analogous to C2 and C3 (Rego et al., 2016b). Further domain mutagenesis studies, combined with more detailed structural analyses of the Bsp and Als3 adhesins, are needed to determine if Bsp-Als3 interactions and SspB-Als3 interactions occur via a common mechanism.

In conclusion, the data represented in this chapter suggest that the synergistic promotion between GBS and *C. albicans* was due to direct physical interactions between GBS and the *C. albicans* cell surface protein, Als3. AgI/II proteins on the surface of GBS contribute to this interaction but other, as of yet unidentified, GBS proteins are also involved.

Chapter 5 Host response to GBS-*C. albicans* interactions

5.1 Introduction

The previous work indicated that *C. albicans* and GBS interact in a way that synergistically enhances the capacity of both microorganisms to associate with vaginal epithelium. A primary mechanism by which microbes may achieve this is by modulation of host responses in a way that makes the VECs more permissive to microbe engagement (Xu et al., 2014a). Host responses to the microbes may also have important implications for the outcomes of these initial interactions in the context of colonisation and disease. Examples of modulation of host responses by *C. albicans-Streptococcus* spp. have been documented. Coinfection of a mouse model of oral infection with *C. albicans* and *S. oralis* was found to induce a strong host inflammatory response. In this case, the most commonly upregulated genes related to neutrophil response and cytokine activity (Xu et al., 2014b). Cytokines involved in neutrophil activity, such as IL-17, IL-1α and IL-1β were induced to elevated levels, facilitating infiltration of neutrophils. This led to an enhanced frequency and severity of oral candidiasis plaques and facilitated systemic spread of *C. albicans* in a mouse model of infection (Xu et al., 2014b).

5.1.1 Host responses to *C. albicans* within the GU tract

Neutrophils are the predominant immune cell in the vaginal tract (Nandi and Allison, 1993), and have been found to be most responsible for killing of *C. albicans* (Edwards et al., 1987;Gazendam et al., 2014;Urban et al., 2009). This correlates with reports of neutropenic patients more commonly experiencing systemic *Candida* disease (Martino et al., 1989). Nonetheless, neutrophils have also been described as more susceptible to killing by *C. albicans* than other immune cells, such as macrophages (Rudkin et al., 2013). Furthermore, despite the role of neutrophils in killing *C. albicans*, rather than clearing the infection, neutrophil infiltration into oral thrush lesions has been found to exacerbate symptoms in the host, and this observation has been extended to vaginal thrush (Yano et al., 2010;Dongari-Bagtzoglou et al., 2009;Xie et al., 2012).

It has been suggested that vaginal (and oral) epithelial cells are directly capable of fungistatic, but not fungicidal, activity in response to *C. albicans* (Nomanbhoy et al., 2002). Oral epithelial cells have been shown to constrain blastospore and hypha growth,

although this was dependent upon cell-fungal contact and was not achieved by saliva or cell supernatant (Steele et al., 2000). Growth inhibition was also dependent on the immune status of the host, as oral epithelium sampled from HIV-infected individuals was incapable of inhibiting *Candida* growth (Steele et al., 2000). A study using mouse VECs found that vaginal cells were able to inhibit growth of *C. albicans* at a MOI of as low as 1:1, but only exhibited a limited amount of fungal killing (Steele et al., 1999). Again, inhibition was not mediated by cell supernatant alone, indicating a direct cell-cell contact mechanism (Steele et al., 1999). Oestrogen reduces the ability of VECs to control *C. albicans* growth, which may explain why woman who suffer from VVC are more susceptible to bouts of symptomatic disease during the luteal phase of menstruation and during pregnancy (Fidel et al., 2000). VECs are also able to produce S100 alarmins in response to *C. albicans*, which have been identified as neutrophil chemoattractants within the vaginal tract (Yano et al., 2014;Yano et al., 2012).

A hallmark of vaginal thrush is an upregulation of inflammatory pathways. Roselletti et al. (2017) highlighted the presence of IL-1β and IL-8, which correlated with an influx of neutrophils (Roselletti et al., 2017). Another study found that reconstituted vaginal epithelium produced IL-1α, IL-1β, IL-6, IL-8, IL-10, G-CSF and IFN-γ following infection with *C. albicans* (Schaller et al., 2005). IL-1α, IL-1β, IL-17 and IL-23 were significantly increased in expression in a model of murine *C. albicans* vaginal infection when compared against control mice (Shroff et al., 2018), and mice were found to be protected from a *C. albicans* infection by a competent IL-17 pathway, which required IL-23 secretion (Gladiator et al., 2013). Of note, *C. albicans* mutants which lacked expression of secreted aspartyl proteinases (SAP) 1 or 2 were less able to induce cytokine production and cause tissue damage (Schaller et al., 2005), implicating these SAPs as modulators of the host immune response. Additionally, injection of SAP2 into the vagina of mice induced an influx of neutrophils and secretion of IL-1β (Pericolini et al., 2015), while *C. albicans* SAP-2 and -6 induced production of IL-8 and MIP-2 by VECs, both of which are neutrophil chemoattractants (Gabrielli et al., 2016). Proinflammatory cytokines such as IL-1α, IL-1β and IL-8 were induced from VECs in a dose-dependent manner by the *C. albicans* virulence factor candidalysin (Richardson et al., 2017). Candidalysin was found to activate c-Fos and MAPKs, and there was a marked reduction in VEC damage and neutrophil infiltration in mice challenged with a candidalysin-deficient strain (Richardson et al., 2017).

Oral and vaginal epithelial cells are known to be able to distinguish which fungi are commensal and which are pathogenic through MAPK, c-Fos and MKP1 signalling (Moyes et al., 2010;Moyes et al., 2011;Moyes et al., 2012). This seems, in part, to be related to whether fungi are present as blastopores or hyphae, with one study outlining how a hypha-deficient strain was a competent coloniser, yet did not induce cell damage (measured by LDH release) or immunopathology (measured by neutrophil infiltration and proinflammatory cytokine production) (Moyes et al., 2010). Stimulation of VECs with *C. albicans* hyphae leads to activation of p38-MAPK and ERK1/2 signalling pathways, causing upregulation of proinflammatory cytokines (Moyes et al., 2011). *C. albicans* also stimulated intracellular signalling events via activation of NF-kappa-B; however this occurred earlier in infection and was independent of *C. albicans* morphology (Moyes et al., 2011). NF-kappa-B is thought to be activated upon recognition of *C. albicans* cell wall constituents such as chitin, β-glucan and mannan. Candidalysin has been shown to be able to lyse oral epithelial cells, as well as stimulate intracellular signalling pathways, including MAPKs (Moyes et al., 2016).

5.1.2 Host responses to GBS within the GU tract

Similar to *C. albicans*, GBS infection can promote the release of proinflammatory cytokines. Mice models have shown that when injected with GBS intravenously or interperitoneally, proinflammatory cytokines including IL-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ are generated (Ernst et al., 2013;Puliti et al., 2002;Rosati et al., 1998). *In vitro*, GBS has been shown to induce IL-1α, IL-1β, IL-8, IL-23 and IL-36γ expression following incubation with VECs, endocervical and ectocervical cells (Patras et al., 2013;Patras et al., 2015a). Furthermore, GBS has been found to stimulate expression of IL-8 in A549 lung epithelial cells in a dose- and time-dependent manner (Doran et al., 2002).

Proinflammatory cytokines are also important in the context of protecting against neonatal GBS infection. IL-12 and IL-18, which are capable of inducing IFN-γ, as well as IFN-γ itself, have been shown in models of neonatal infection to protect the host from GBS infection (Mancuso et al., 1997). Mice pups had a significantly higher number of bacteria in the blood, as well as a higher likelihood of death due to infection, if they were pre-incubated with anti-IL-12 antibodies prior to GBS challenge, while treatment with recombinant IL-12 reversed these effects (Mancuso et al., 1997;Cusumano et al.,

2004;Cusumano et al., 1996). Similarly, neonatal mice with GBS sepsis had significantly increased morbidity and mortality when pre-incubated with anti-IL-18 antibody (Cusumano et al., 2004). Treatment of the mice with recombinant IL-18 increased survival rates and lowered the CFU of GBS in the blood. The protective effects conferred by recombinant IL-12 or IL-18 when administered prophylactically were outweighed by pre-incubation with anti-IFNγ antibodies, suggesting that the role of IL-12 and IL-18 in host defence against GBS was due to upregulation of IFN-γ (Mancuso et al., 1997;Cusumano et al., 2004). To support this, inoculation of neonatal mice with recombinant IFN-γ after GBS challenge enhanced chances of survival and decreased levels of GBS in the blood (Cusumano et al., 1996).

Neutrophils are important components of the immune response for clearance of GBS. Neutrophils are recruited to sites of infection upon secretion of IL-1β, as this induces expression of chemokines CXCL1, CXCL2, KC and MIP-1α (Biondo et al., 2014a;Biondo et al., 2014b). This is magnified by neutrophils expressing IL-1β themselves, causing a positive feedback loop (Mohammadi et al., 2016). IL-1β-deficient mice were much more susceptible to GBS infection, and were killed by levels of GBS that WT mice survived, with GBS exhibiting an enhanced ability to systemically spread (Biondo et al., 2014a). Furthermore, mice deficient in the IL-1 receptor had not only reduced neutrophil infiltrate, but also showed a reduction in GBS clearance (Biondo et al., 2014b). Nonetheless, GBS can counteract neutrophil-mediated clearance through the expression of serine protease CspA. This cleaves CXC chemokines, including GRO-α (CXCL1), GRO-β (CXCL2), and GRO-γ (CXCL3), preventing these chemokines from attracting and activating neutrophils (Bryan and Shelver, 2009). In addition to neutrophils, macrophages can play an important role in controlling pathogenesis of GBS within the GU tract. Protein kinase D1 is activated in macrophages by both live and killed GBS. This is necessary for triggering activation of MAPKs and NF-kappa-B, leading to production of proinflammatory cytokines and chemokines in the vaginal tract (Upadhyay et al., 2017).

GBS is recognised by Toll-like receptors (TLRs) on the surface of host cells. Activation of TLRs leads to activation of p38 MAPK and NF-kappa-B, which are involved in triggering secretion of proinflammatory cytokines and reactive oxygen species (ROS) from immune cells (Henneke et al., 2002). The single-stranded RNA produced by GBS is recognised by MyD88, a TLR adaptor protein, on macrophages and causes release of proinflammatory cytokines as a result (Deshmukh et al., 2011). Moreover, the activation of p38 and NF-

kappa-B by TLRs in response to GBS is dependent on expression of MyD88 (Henneke et al., 2002). Again, however, GBS can evade such immune responses. The pore-forming toxin β-H/C has been shown to activate p38 in macrophages to induce expression of IL-10, which is anti-inflammatory and immunosuppressive, and macrophages deficient in p38 are more effective in mounting a robust anti-GBS response (Bebien et al., 2012). GBS two-component regulator CovR also influences pathogenicity of GBS in terms of vaginal adherence, invasion and host response (Patras et al., 2013). A CovR null mutant exhibited increased binding to VECs, but decreased invasion, as well as inducing higher levels of chemokines including IL-8 from host cells *in vitro* (Patras et al., 2013). Interestingly, the WT strain persisted longer within the vaginal tract of mice than the CovR mutant, possibly due to the dampened proinflammatory (IL-8) response.

Alongside modulation of host cytokine responses, GBS can induce epithelial exfoliation via integrin and β-catenin signalling (Vornhagen et al., 2018). Transepithelial resistance was shown to be significantly reduced when VECs were incubated with GBS, and epithelial cells demonstrated increased permeability to fluorescein and nanoparticles after infection with GBS, meaning that GBS affects the barrier function of VECs. GBS were consequently more able to migrate across the epithelium into the lower compartment of a transwell. In a mouse model of vaginal infection, VECs were observed to exfoliate 48 h after GBS challenge. Increased permeability and a decrease in barrier function are hallmarks of epithelial-mesenchymal transition (EMT), and this was the mechanism found to be the cause of GBS-induced exfoliation, with VECs expressing a reduced level of E-cadherin in favour of N-cadherin. Exfoliated cells also had changes in transcription of EMT genes such as SNAIL1 (Vornhagen et al., 2018). β-catenin signalling is involved in EMT, and blocking β-catenin prevented epithelial exfoliation. GBS-infected cells had higher levels of active integrin than control cells, with competitive inhibition of integrin signalling reducing VEC exfoliation. As such, integrin signalling was hypothesised to be the mechanism for activation of β-catenin (Vornhagen et al., 2018). Although studies of other GU pathogens have shown that vaginal epithelial exfoliation is a host defence mechanism which decreases the bacterial load (Muenzner et al., 2005;Muenzner et al., 2010), epithelial exfoliation induced by GBS actually enhanced numbers of colonising bacteria in the uterus, suggesting that exfoliation facilitated GBS ascension of the vaginal tract (Vornhagen et al., 2018). This was supported using a mouse model of ascending GBS infection, whereby pregnant mice were infected with GBS and treated with either recombinant α1β1 integrin (to competitively inhibit integrin

signalling) or PBS. Treatment with α1β1 integrin reduced GBS ascension, as shown by reduced bacterial load in the placenta, uterus and foetal tissues, and there was a reduction in preterm birth or stillbirth (Vornhagen et al., 2018). This could suggest a role in blocking these signalling pathways in order to reduce the morbidity and mortality associated with ascending GBS infection.

Given the well-characterised capacity for GBS and *C. albicans* to modulate their host during monospecies infections, it was hypothesised that the synergistic interactions of these microbes with VECs may, in turn, affect the host response to these microorganisms. To investigate this in greater detail, studies were performed to explore the relationship between GBS-*C. albicans* interactions and neutrophil chemotaxis, proinflammatory cytokine production and host protein modulation.

5.2 Results

5.2.1 Neutrophil chemotaxis studies

Although it had been identified that there was a synergistic partnership between *C. albicans* and GBS, it remained to be established what effect this had on the host. To begin to examine the host response, a neutrophil chemotaxis model was used. These studies were performed in the laboratories of Dr Rebecca Hall (Univ Birmingham). Neutrophils were isolated from human blood, stained with syto-13 and adhered to the viewing chamber of an Ibidi chemotaxis slide. To the left-hand chamber of the slide was added K-SFM, while 1:2 diluted spent medium from VEC association assays was added to the right-hand chamber. K-SFM was used as a negative control, and 200 nM *N*-formylmethionine-leucine-phenylalanine (FMLP) as a positive control. FMLP is a chemoattractant, first characterised from *E. coli* (Marasco et al., 1984).

Chemotaxis slides were transferred to a microscope and images were taken of the viewing chambers every 2 min for 1 h, i.e. 31 images were taken per field. These images were analysed for neutrophil movement using the Fiji Trackmate plugin (ImageJ), data from which were further analysed with the Ibidi Chemotaxis and Migration program (Ibidi®). Due to the orientation of the microscope camera, the neutrophil gradient was

along the y axis. To maximise the variability assessed by the assays, a different neutrophil donor was used with each VEC assay spent medium replicate.

Representative chemotaxis plots were generated to display the difference in neutrophil activity and consequent movement (Figure 5-1). The start point of each neutrophil was normalised to the origin of the graph, to better present the direction of movement of each neutrophil. The green dot at the centre of each neutrophil population represents the centre of mass, i.e. the average movement of all of the neutrophils which have been tracked. If there was a positive chemotactic effect (i.e. neutrophils were moving towards the test sample), the centre of mass would move down the y axis. This was observed for the positive control, FMLP, alongside spent medium from VECs incubated with each of the three GBS strains or with *C. albicans*+COH1 (Figure 5-1). The chemoattractant effect was most noticeable with COH1, which may account for a chemotactic effect only being seen using spent medium from dual-species assays with COH1 rather than with NEM316 or 515 (Figure 5-1). The chemotaxis plots for spent medium from *C. albicans* alone showed some neutrophil activation, and this was visibly more than was observed with the negative VEC-only spent medium control. However, there was no direction, indicating that the neutrophils were stimulated but were not attracted to the source of stimulation. Similar plots were observed for neutrophils exposed to spent medium from *C. albicans*+NEM316 and *C. albicans*+515 (Figure 5-1); however the neutrophils appeared to show more overall activity than the *C. albicans* plot. No neutrophil activity or chemotaxis was observed for the VEC plot, which showed neutrophils clustered around the origin of the graph, indicating that the majority of neutrophils were stationary throughout the assay (Figure 5-1). A similar plot was generated with neutrophils exposed to K-SFM (data not shown).

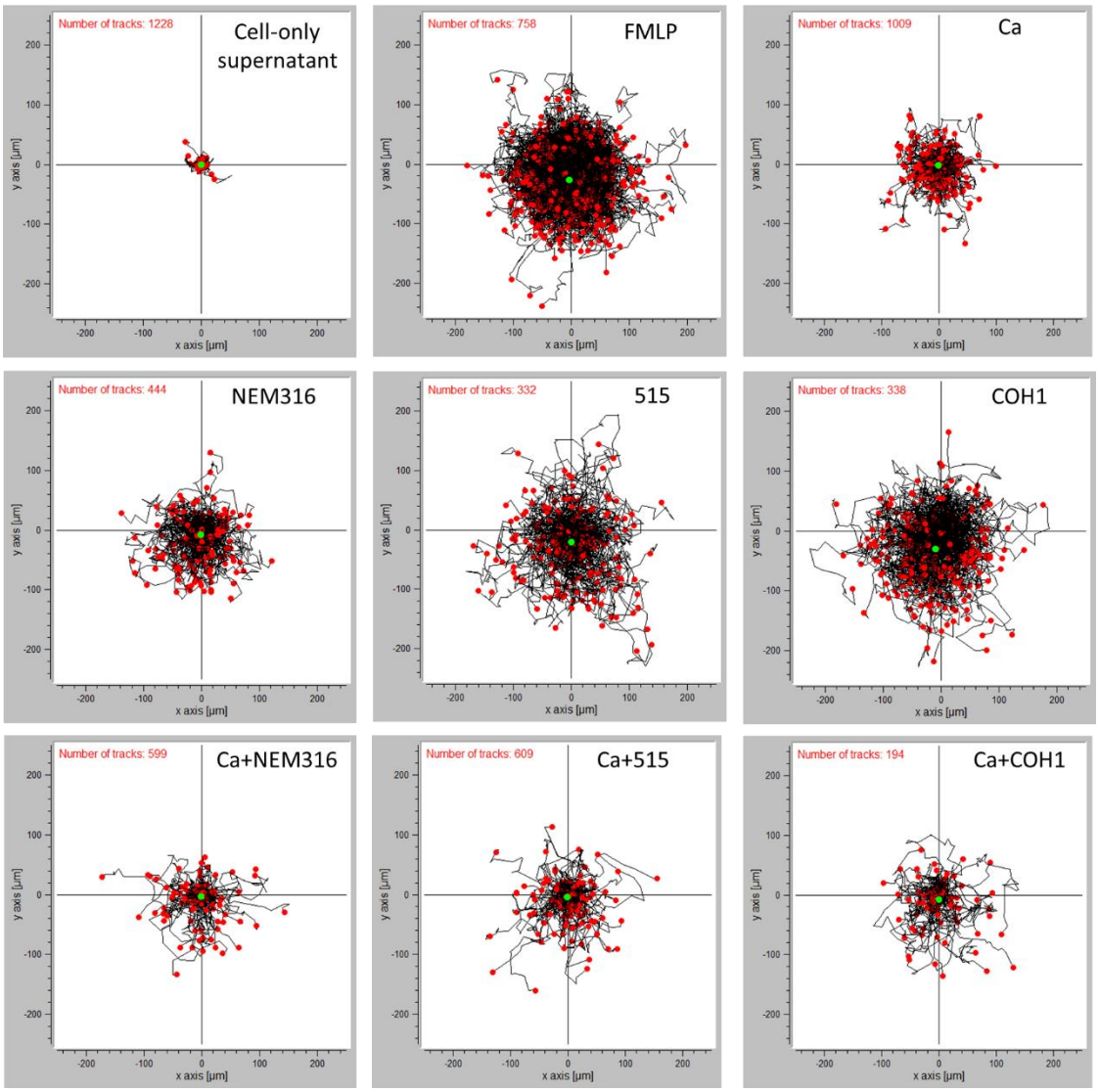

Figure 5-1 Plots of neutrophil chemotaxis in response to conditioned media from VEC association assays.

VECs were incubated with *C. albicans* (MOI 2.5) for 6 h, or GBS (MOI 2.5) for 5 h. Alternatively, VECs were incubated with *C. albicans* for 1 h followed by addition of GBS and incubation for a further 5 h. Conditioned media were collected from well plates and microbes were pelleted for removal. Neutrophils were adhered to Ibidi chemotaxis slides. K-SFM, 200 nM FMLP or conditioned media, as indicated, were added and slides were imaged with a microscope every 2 min over 1 h (31 time points in total). Neutrophils were tracked using Fiji TrackMate, from which data were inputted into Ibidi Chemotaxis and Migration tool to generate chemotaxis plots. Red dots indicate neutrophil end points, while green dots indicate the centre of mass of the neutrophil population. Studies were performed on 4 occasions. Data are presented from one representative experiment.

Neutrophil tracks were analysed for centre of mass and forward migration (Figure 5-2), and for directionality, distance and velocity (Figure 5-3). For all parameters tested, neutrophils did not respond any differently to blank K-SFM medium than they did to conditioned media from VECs (Figure 5-2 and Figure 5-3). Centre of mass is the average movement of the population of neutrophils across the duration of the assay, while

forward migration index (FMI) is a measure of whether the neutrophils are moving randomly. Strong chemotaxis effects are described as having large FMI parallel to the gradient (FMI$_{\parallel}$, Figure 5-2D) and a perpendicular FMI that is close to zero (FMI$^{\perp}$, Figure 5-2C). A strong chemotactic effect was seen in the presence of GBS; when VECs were incubated with GBS, both centre of mass$_{\parallel}$ and FMI$_{\parallel}$ were found to be large. However, considerably less chemotaxis was observed in response to dual-species-infected VECs (Figure 5-2B, D). The chemotactic effects of conditioned media from VECs incubated with GBS were found to be significantly different to those of the VEC-only control conditioned media for both centre of mass and FMI parallel to the gradient (Figure 5-2B, D), while the effects of conditioned media from VECs incubated with only *C. albicans* or both species were not significantly different. For centre of mass$_{\parallel}$, conditioned media recovered from VECs incubated with *C. albicans* or both microorganisms, with the exception of *C. albicans*+COH1, were significantly different from the FMLP positive control (Figure 5-2B). For FMI$_{\parallel}$, this was only true for conditioned media from VECs incubated with *C. albicans* or *C. albicans*+NEM316 (Figure 5-2D). This implied an inherent difference in the way VECs responded to GBS alone compared to GBS with *C. albicans*. Little neutrophil movement was observed perpendicular to the gradient (Figure 5-2A, C) for either centre of mass or FMI, indicating that neutrophils predominantly moved in the direction of the gradient, if at all. This reinforces the representative chemotaxis plots (Figure 5-1), indicating that the neutrophils generally responded to positive signals.

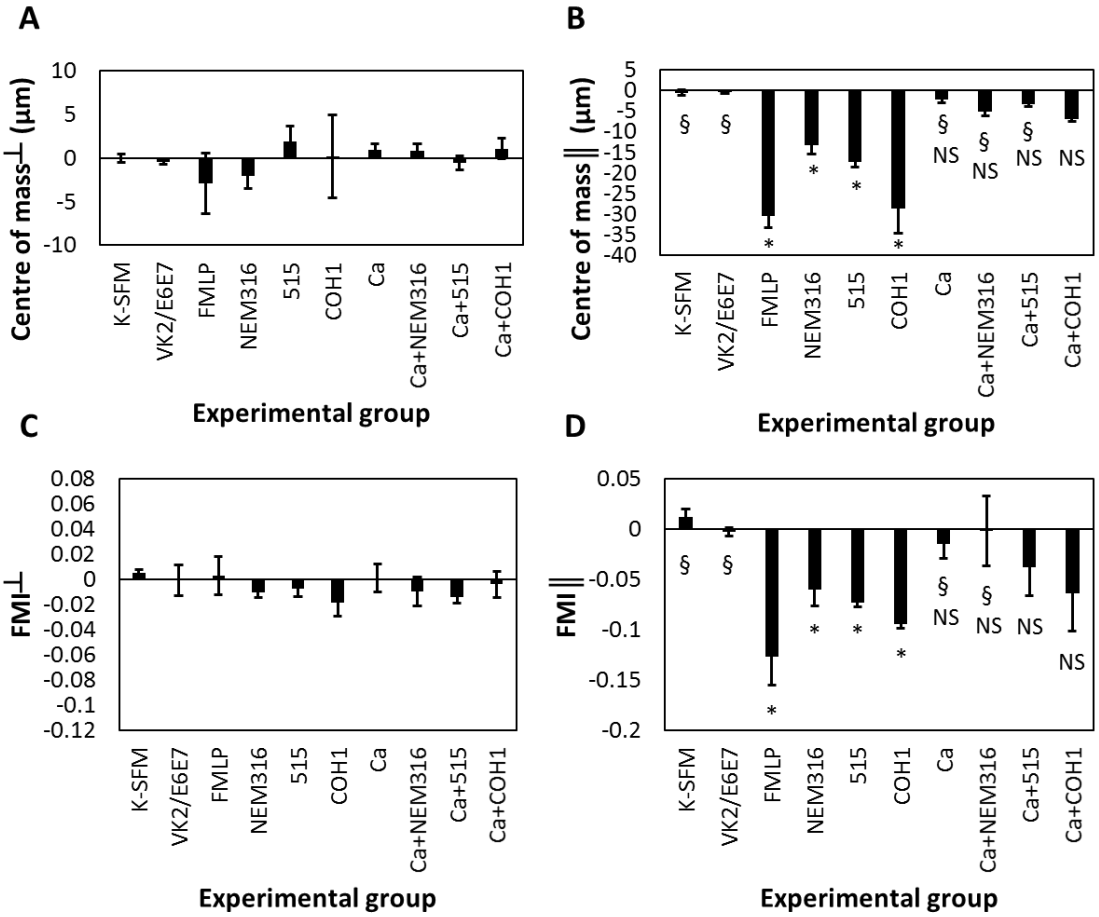

Figure 5-2 Chemotaxis of neutrophils in response to conditioned media from VEC association assays.

VECs were incubated with *C. albicans* (MOI 2.5) for 6 h, or GBS (MOI 2.5) for 5 h. Alternatively, VECs were incubated with *C. albicans* for 1 h followed by addition of GBS and incubation for a further 5 h. Conditioned media were collected from well plates and microbes were pelleted for removal. Neutrophils were adhered to Ibidi chemotaxis slides. K-SFM, 200 nM FMLP or conditioned media from VEC assays was added and slides were imaged every 2 min over 1 h (31 time points in total). A, B) centre of mass of the population of neutrophils; C, D) forward migration index of neutrophil population. A, C) examine these parameters on the x axis (perpendicular to the gradient); B, D) examine these parameters on the y axis (parallel to the gradient). Data are presented as mean ± SEM. * indicates $P<0.05$ relative to VK2/E6E7 control, NS indicates $P>0.05$ relative to VK2/E6E7 control, § indicates $P<0.05$ relative to FMLP positive control as determined by independent samples Kruskal Wallis; n=4.

Further parameters that the neutrophils were tested for were velocity and directionality, a measure of how direct the neutrophils' movement was (Figure 5-3A, B). Additionally, the accumulated and Euclidean distance were tested (Figure 5-3C, D). Accumulated distance was defined as the total distance covered by the neutrophils, while Euclidean distance was defined as the distance between the neutrophil start and end point. Similar to the pattern seen for FMI‖ and centre of mass‖ in Figure 5-2, there were no significant differences between neutrophil response to the VEC-only conditioned media when compared to conditioned media from VEC infected with either

C. albicans or *C. albicans*+GBS for velocity (Figure 5-3B), accumulated or Euclidean distance (Figure 5-3C,D). However, there was also no significant difference for the conditioned media from VEC infected with NEM316 for accumulated and Euclidean distance (Figure 5-3C, D), suggesting an attenuated neutrophil response to NEM316 which may enable persistence. Additionally, for directionality only conditioned media from cells infected with just *C. albicans* or *C. albicans*+NEM316 were not significant when compared against the neutrophil response to VECs. The other experimental groups exhibited similar levels of directionality (Figure 5-3A).

For each of these parameters, there were no significant differences between the neutrophil response to FMLP compared to the GBS-only groups, while there were significant differences between FMLP and *C. albicans*-only or the negative controls. However, for directionality, there were also no significant differences between FMLP and *C. albicans* with any of the GBS strains. For velocity, there was no significant difference between FMLP and *C. albicans*+515. Meanwhile, for both accumulated and Euclidean distance, all of the dual-species groups were significantly different to FMLP. This further suggests that, in general, when GBS was incubated with VECs alone, release of chemoattractants was stimulated, while when GBS was co-incubated with *C. albicans*, this response was attenuated.

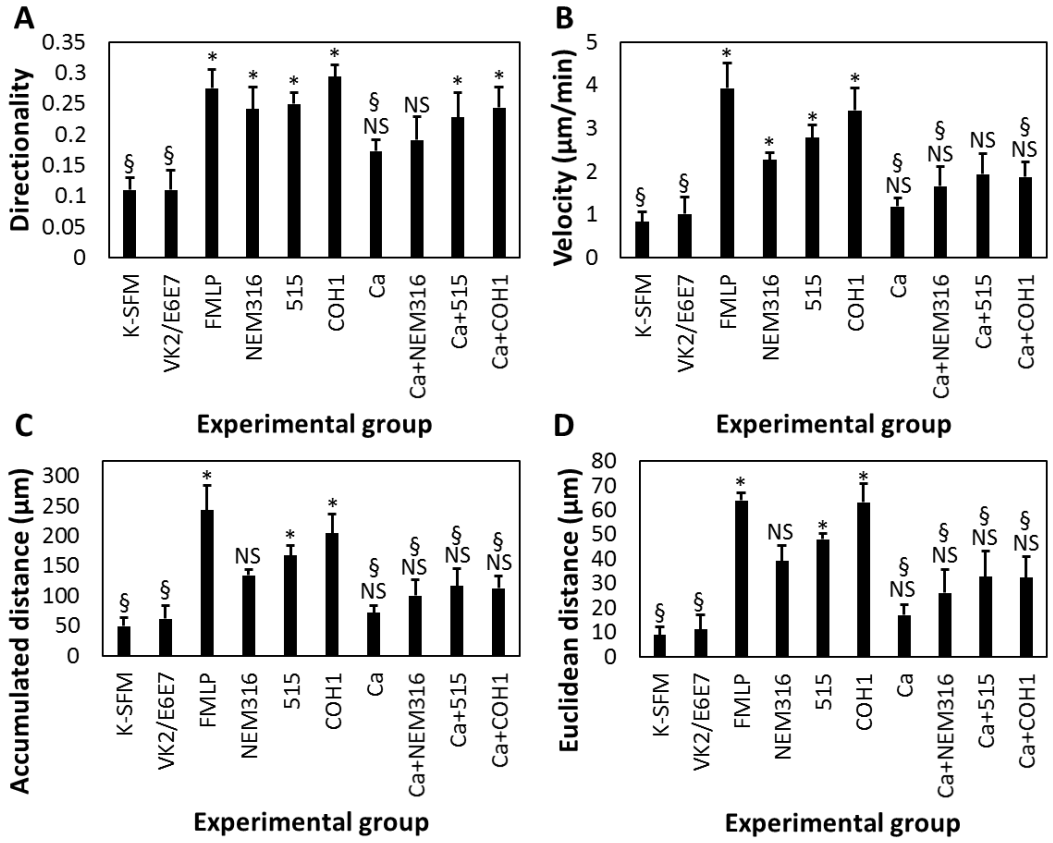

Figure 5-3 Chemotaxis of neutrophils in response to conditioned media from VEC association assays.

VECs were incubated with *C. albicans* (MOI 2.5) for 6 h, or GBS (MOI 2.5) for 5 h. Alternatively, VECs were incubated with *C. albicans* for 1 h followed by addition of GBS and incubation for a further 5 h. Supernatant was collected from well plates and microbes were pelleted for removal. Neutrophils were adhered to Ibidi chemotaxis slides. K-SFM, 200 nM FMLP or conditioned media from VEC assays were added and slides were imaged with a microscope every 2 min over 1 h (31 time points in total). A) Directionality or B) velocity of the population of neutrophils; C) accumulated or D) Euclidean distance of the neutrophil population. Data are presented as mean ± SEM. * indicates $P<0.05$ relative to VK2/E6E7 control, NS indicates $P>0.05$ relative to VEC control, § indicates $P<0.05$ relative to FMLP positive control as determined by independent samples Kruskal Wallis; n=4.

5.2.2 Proteome analysis

To investigate, on a protein level, the VEC response to GBS-*C. albicans* interactions, mixed-species association assays were carried out using GBS strain NEM316. Cells were then lysed by recovery into 8 M urea, followed by tryptic digestion of the total protein. Peptides were analysed by tandem mass tagging and subsequent mass spectrometry, and identified using the FASTA database. Peptides generated spectra which could be used to identify which species they belonged to, alongside the calculated theoretical number of amino acids, molecular weight and isoelectric point. Following identification, further analysis excluded proteins which were detected in only one of the experimental infection replicates or that were not changed in expression relative to the VEC-only control. Proteins were considered upregulated if expression was at least 2-fold higher than the VEC-only control or considered downregulated if expression was 2-fold or more reduced (i.e. expression at 0.5-fold or lower). These shortlisted proteins were then further analysed using the Protein ANalysis THrough Evolutionary Relationships (PAnThER) database, which collects Gene Ontology (GO) annotations for each protein. Some of the proteins had multiple annotations, while others were uncharacterised or had limited information available, which restricted the analysis (Table 5-1). The three GO terms 'biological process', 'cellular component', and 'molecular function' were investigated as well as the additional PAnThER terms 'protein class' and 'pathway'. These were selected as they are believed to be effective for classifying proteins based on their functions (Thomas et al., 2003;Mi et al., 2013).

Table 5-1 Overview of VEC proteins that were upregulated or downregulated in response to infection

Sample Group	Protein Group	Total Number Proteins	Proteins Not Found	GO: Biological Process	GO: Molecular Function	GO: Cell Component	GO: Protein Class	GO: Pathway
Dual-species	Upregulated	136	1	175 hits	81 hits	95 hits	72 hits	43 hits
	Downregulated	299	7	354 hits	186 hits	200 hits	196 hits	170 hits
C. albicans-only and *C. albicans*+GBS	Upregulated	146	87	98 hits	39 hits	52 hits	36 hits	43 hits
	Downregulated	150	77	103 hits	46 hits	58 hits	35 hits	19 hits
GBS-only and *C. albicans*+ GBS	Upregulated	8	4	0 hits	0 hits	0 hits	0 hits	0 hits
	Downregulated	6	2	6 hits	2 hits	4 hits	4 hits	0 hits

5.2.2.1 VEC response to GBS

There were 30 VEC proteins significantly altered in response to GBS between the VEC-only control and VECs exposed to GBS monospecies infection. Few proteins were directly associated with what is known regarding the pathogenesis of GBS. The most common theme amongst upregulated proteins was the cytoskeleton, including cytokeratin 4, which has been described as a binding partner for the GBS adhesin Srr1 (Sheen et al., 2011). Of the downregulated proteins, the most common themes were transcriptional regulation and cytoskeletal organisation (Appendix 1 and Appendix 2).

Somewhat surprisingly, there were only 14 VEC proteins which were significantly altered in expression between the VEC-only control and VECs exposed to GBS monospecies or dual-species infection: 8 upregulated and 6 downregulated. Due to the small number of proteins altered in expression within this group, it is not possible to draw many major conclusions from these data. Although few proteins correlated with pathogenesis, the particularly steep increase in expression of actin gamma 1 (from 9.5 in monospecies to 100 in dual-species infection, Table 5-2) may suggest that the presence of *C. albicans* can exacerbate cytoskeletal rearrangements induced by GBS. Likewise, the 10-fold increase in abundance of heat shock protein 90Af (from 2.7 to 28.3, Table 5-2) may indicate an elevated stress response in VECs following dual-species infection relative to monospecies infection. This may reflect evidence from previous chapters that VECs are exposed to increased numbers of GBS when in the presence of *C. albicans*. Three other cytoskeleton proteins were upregulated, all of which were keratins (Table 5-2). This suggests major upregulation of cytoskeletal proteins, which GBS may be able to target as sites for adhesion to the epithelial cells. Among the downregulated proteins, there was no clear theme in protein function and, aside from one protein involved in reorganisation of the cytoskeleton, there was not a clear link between function and what is known about the pathogenesis of these microorganisms (Table 5-3).

Table 5-2 Overview of proteins that were upregulated only in the GBS-infected and *C. albicans*+GBS-infected VECs relative to the VEC-only control.

Protein code	Protein name	Protein function	Relative abundance (VECs + GBS) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
V9HVZ7	Actin gamma 1	Actin	9.483	100
Q58FF9	Heat shock protein 90Af	Protein folding	2.747	28.277
Q9BSM1	Polycomb group RING finger protein 1	Transcriptional repressor	2.494	2.529
Q86Y38	Xylosyltransferase 1	Biosynthesis of GAGs	2.492	3.558
H0Y459	Nuclear receptor corepressor 1	Chromatin condensation	2.267	2.267
P35527	Keratin, type I cytoskeletal 9	Keratin	2.265	2.435
Q86Y46	Keratin, type II cytoskeletal 73	Keratin	2.099	2.266
H6VRG1	Keratin 1	Keratin	2.011	2.328

Table 5-3 Overview of proteins that were downregulated only in the GBS-infected and *C. albicans*+GBS-infected VECs relative to the VEC-only control.

Protein code	Protein name	Protein function	Relative abundance (VECs + GBS) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
B2R6X5	Heat shock 70kDa protein 6	Folding of proteins	0.285	0.257
Q66K64	DDB1- and CUL4-associated factor 15	Ubiquitination	0.397	0.01
Q15363	Transmembrane emp24 domain-containing protein 2	Vesicle protein trafficking	0.484	0.433
Q5HYK7	SH3 domain-containing protein 19	Regulates organisation of the cytoskeleton	0.488	0.476
P55060	Exportin-2	Export receptor	0.498	0.425
B4DY32	Asparagine synthetase	Asparagine synthesis	0.498	0.463

5.2.2.2 VEC response to *C. albicans*

A total of 68 VEC proteins were modulated by *C. albicans*, but not in the presence of GBS: 28 upregulated and 34 downregulated (see Appendix 3 and Appendix 4 for complete lists). B-cell lymphoma 3 protein was upregulated in response to *C. albicans*-only infection, and has a role in activation of NF-kappa-B, while another upregulated protein was found to be a subunit of NF-kappa-B, which could suggest an increase in host defence-related signalling. Another key theme among the upregulated proteins was cytoskeletal organisation, while for the downregulated proteins this was transcriptional regulation. Overall, however, there was no strong correlation with the precise pathways known to be targeted by *C. albicans*.

A total of 146 VEC proteins were upregulated and 150 were downregulated in response to *C. albicans*, independent of the presence of GBS (i.e. were identified for both the *C. albicans*-only sample and the *C. albicans*+GBS sample) (Table 5-1; see Appendix 5 and Appendix 6 for complete lists). A high proportion were not annotated in the PAnThER database, but available proteins were assessed against the three GO terms as well as two additional PAnThER categories. 'Biological process' comprised 12 categories (Figure 5-4A), 'cellular component' comprised 7 (Figure 5-4B), 'molecular function' comprised 7 (Figure 5-4C), 'protein class' comprised 19 (Figure 5-5) and 'pathway' comprised 38 (Figure 5-6). Among the 'biological process' category, the most significantly overrepresented terms were 'cellular process' and 'metabolic process'. This was seen for both up- and downregulated proteins, although the latter were most abundant. A similar pattern was seen for category 'cellular component', for which the most significantly enriched terms were 'cell part' and 'organelle'. Within the 'molecular function' category, downregulated proteins belonging to the terms 'catalytic activity' or 'binding' were the most abundant. The most enriched, downregulated proteins within the category 'protein class' were assigned across a number of different terms, the top three of which were 'enzyme modulator', 'nucleic acid binding' and 'transcription factor'. The most abundant proteins that were upregulated in this category belonged to the terms 'hydrolase' and 'transporter'. For the category 'pathway', significantly expressed proteins were distributed across numerous terms. However, more proteins were up- than downregulated and of these, proteins associated with terms 'CCKR signaling map', 'gonadotropin-releasing hormone receptor pathway', 'inflammation mediated by CXC/CK signaling' and 'integrin signaling pathway' were the most abundant.

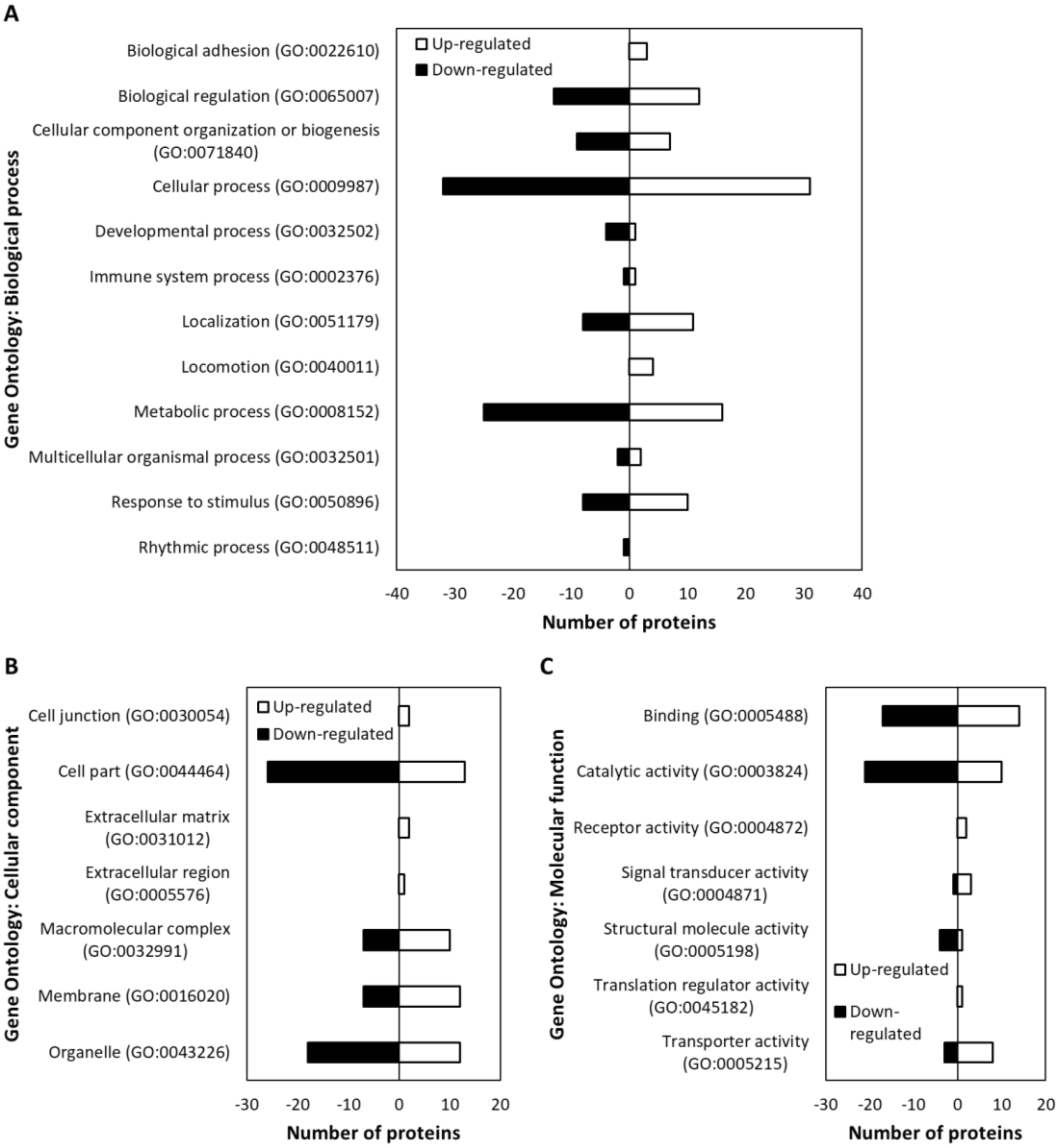

Figure 5-4 Distribution of VEC proteins grouped into GO categories that were significantly altered in *C. albicans* and *C. albicans*+GBS infected cells.

Significantly upregulated proteins, white bars; significantly downregulated proteins, black bars. A) Biological process, B) cellular component, C) molecular function. Proteins included across all terms exhibited ≥2-fold expression difference relative to uninfected VECs; n=2.

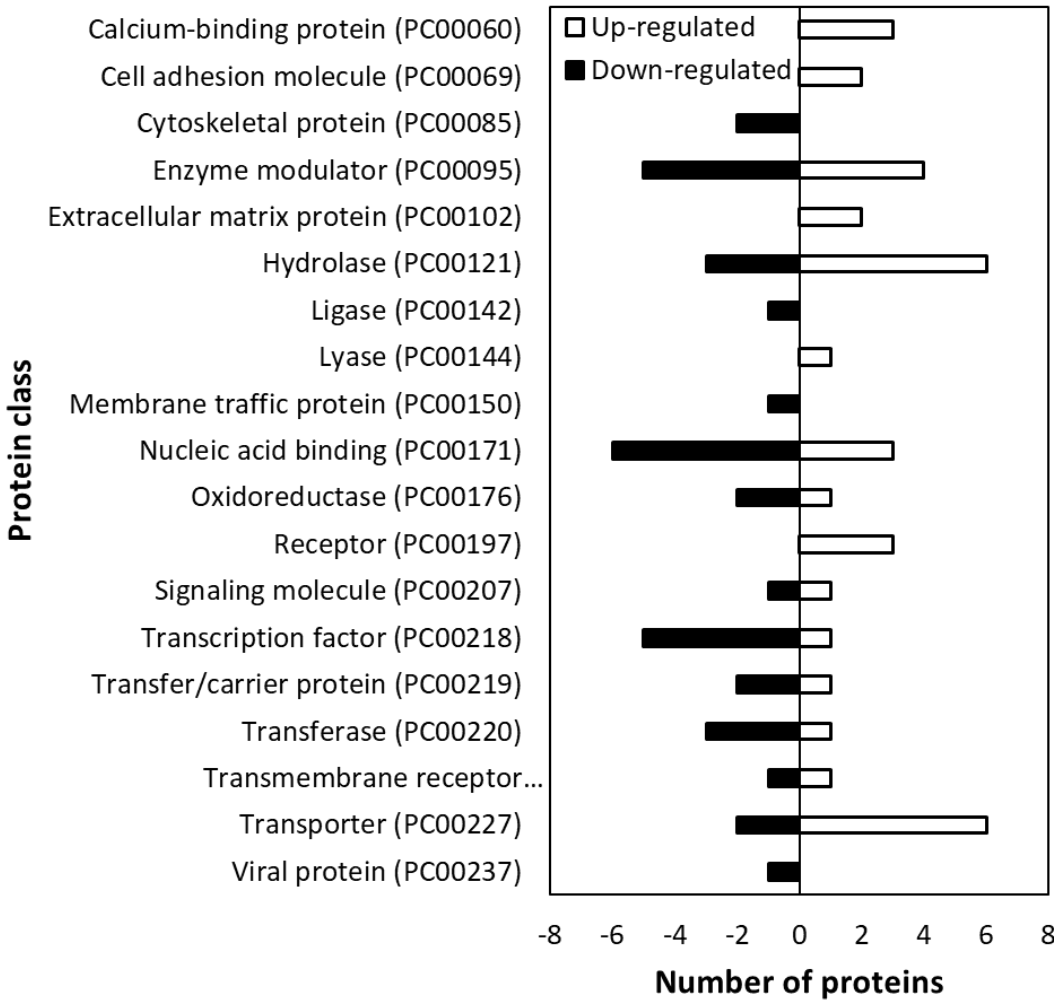

Figure 5-5 Distribution of VEC proteins grouped into the category 'protein class' that were significantly altered in *C. albicans* and *C. albicans*+GBS infected cells.

Significantly upregulated proteins, white bars; significantly downregulated proteins, black bars. Proteins included across all terms exhibited ≥2-fold expression difference relative to uninfected VECs; n=2.

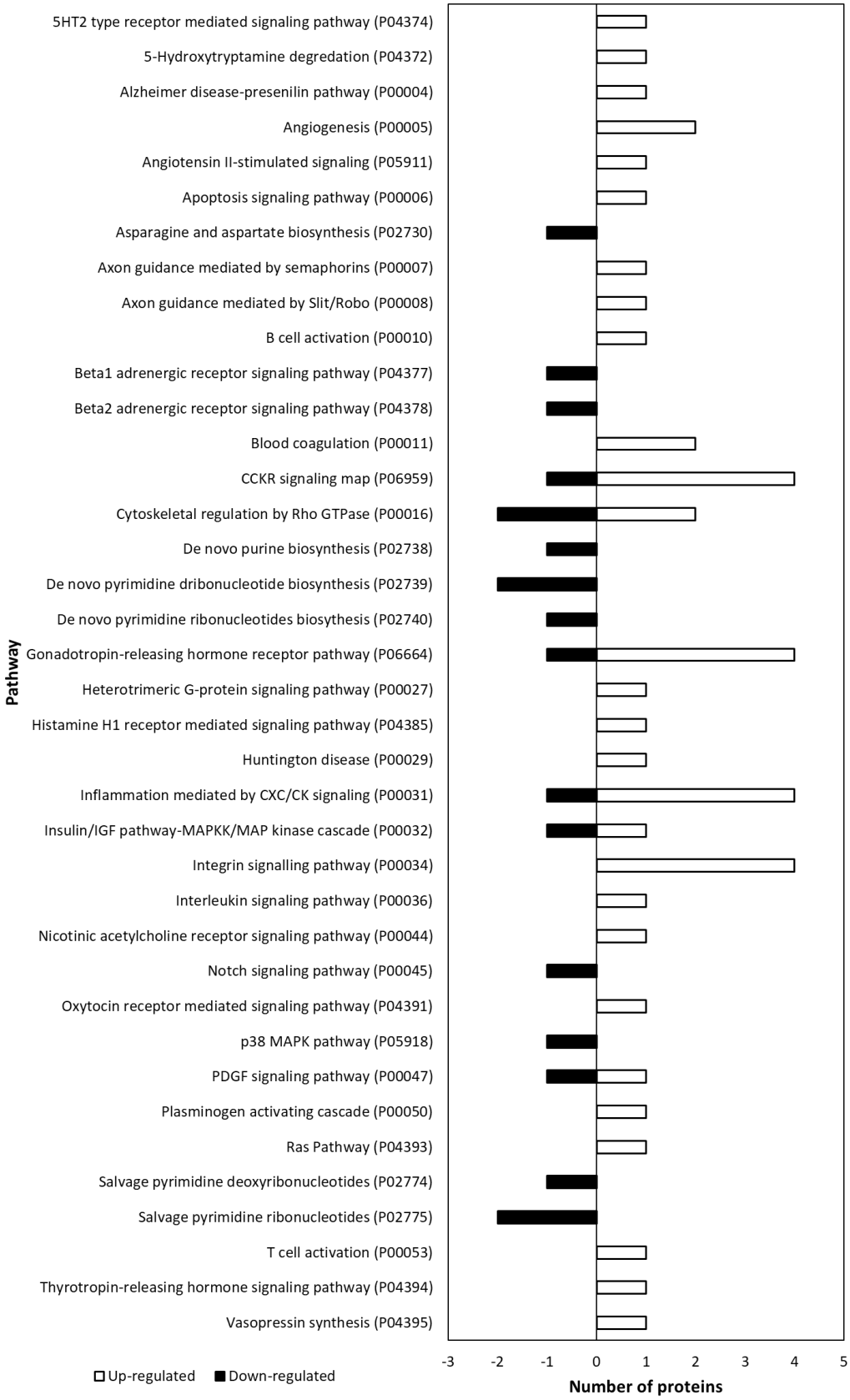

Figure 5-6 Distribution of VEC proteins grouped into the category 'pathway' that were significantly altered in *C. albicans* and *C. albicans*+GBS infected cells.

Significantly upregulated proteins, white bars; significantly downregulated proteins, black bars. Proteins included across all terms exhibited ≥2-fold expression difference relative to uninfected VECs; n=2.

To build on the overview provided by the PAnThER analysis and to try and understand the VEC response to *C. albicans* infection in greater detail, the individual proteins were also investigated. To aid this process, particular focus was given to those proteins that, based on functionality, had potential relevance to the context of microbial infection (e.g. surface-bound proteins, those with antimicrobial properties, major regulators, those that engage with the immune response). Of the proteins which were upregulated in the VECs infected with *C. albicans* alone and *C. albicans*+GBS (Table 5-4), the most abundant protein was an inhibitor of the complement cascade, which inactivates complement components C3b and C4b. Two other abundant proteins, chromogranin and lactoferrin, have antimicrobial activity. Chromogranin is a precursor of five peptides, one of which (catestatin) has antimicrobial activity and acts as a chemoattractant of macrophages (Egger et al., 2008;Briolat et al., 2005;Radek et al., 2008), while another (chromofungin) has antifungal activity (Lugardon et al., 2002). Lactoferrin is found in mucosal secretions and binds strongly to iron, causing a static effect on microbial growth, as well as directly causing cell lysis in a broad range of microbes (Garcia-Montoya et al., 2012). Other common themes seen among the upregulated proteins were proteins associated with organisation of the actin cytoskeleton, such as β-actin and FAK-1, and with cell-cell or cell-ECM adhesion. These included a variety of surface-exposed receptors such as CD44, ICAM-1 and integrin receptors.

Table 5-4 VEC proteins which were upregulated both when VECs were incubated with *C. albicans* and *C. albicans*+GBS which were most relevant to infection

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
B4DRF2	Complement factor I	Regulates complement cascade	5.619	5.113
P02787	Serotransferrin	Iron transport, membrane protein	5.582	5.758
P10645	Chromogranin-A	Antimicrobial peptide	4.833	5.083
LTF	Lactoferrin	Antimicrobial peptide, iron binding	4.235	4.912
P53985	Monocarboxylate transporter 1	Membrane transporter, membrane protein	3.09	3.349
E5RJP0	Focal adhesion kinase 1 (FAK-1)	Cytoskeletal organisation, MAPK activation	3.01	3.727
P05026	Sodium/potassium-transporting	ATPase, cell adhesion, membrane protein	2.935	3.061

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	ATPase subunit beta-1			
A1E282	Beta-actin	Actin	2.803	2.384
SLC16A3	Solute carrier family 16 (Monocarboxylic acid transporters), member 3	Membrane transporter, membrane protein	2.797	2.762
P48509	CD151 antigen	Cell adhesion, integrin binding, membrane protein	2.682	2.812
V9HW63	Epididymis secretory sperm binding protein Li 97n	Regulates NF-kappa-B	2.681	3.219
SET	SET translocation (Myeloid leukemia-associated)	Apoptosis, transcription	2.642	3.015
E7EWM2	Centrosomal protein of 170 kDa	Cytoskeletal organisation	2.634	3.096
P18084	Integrin beta-5	Cell adhesion, membrane protein	2.585	2.484
P08123	Collagen alpha-2(I) chain	Collagen	2.553	2.296
U3KQV3	Unnamed protein	GTPase, membrane protein	2.536	3.125
P51884	Lumican	Collagen binding and organisation	2.522	2.704
PANX1	Pannexin	Component of gap junctions, membrane protein	2.456	2.425
V9HWA9	Epididymis secretory sperm binding protein Li 62p	Complement C3, antimicrobial activity	2.409	2.14
G8JLH6	Tetraspanin	Cell surface protein, membrane protein	2.39	2.063
U3KQE2	Calpain small subunit 1	Cytoskeletal organisation, influences apoptosis proteins, adhesion	2.385	3.808
TNC	Tenascin C (Hexabrachion)	ECM protein	2.339	2.827
F5GZS6	4F2 cell-surface antigen heavy chain	Ion transport, membrane protein	2.323	2.375

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
P54709	Sodium/potassium-transporting ATPase subunit beta-3	ATPase, membrane protein	2.275	2.304
Q59G10	Aldehyde dehydrogenase 1 family, member L1 variant	Regulates apoptosis	2.266	2.472
P19256	Lymphocyte function-associated antigen 3	T cell activation, membrane protein	2.262	2.548
H7BY55	Complement decay-accelerating factor	Inhibits complement cascade, membrane protein	2.23	2.403
B2RAH2	Sodium/hydrogen exchanger	Membrane transporter, expressed in kidney and intestine	2.221	2.302
P16070	CD44 antigen	Mediates cell-cell and cell-matrix interactions, membrane protein	2.208	2.394
E7ESP4	Integrin alpha-2	Cell adhesion, ECM protein receptor, membrane bound	2.177	2.563
Q8NI35	InaD-like protein	May regulate protein targeting, cell polarity and integrity of tight junctions, membrane protein	2.143	2.321
Q9Y4G2	Pleckstrin homology domain-containing family M member 1	Regulation of endosomal trafficking	2.131	3.123
P05556	Integrin beta-1	Cell adhesion, membrane protein	2.106	2.384
Q03405	Urokinase plasminogen activator surface receptor	Plasmin formation, membrane protein	2.104	2.449
Q9Y639	Neuroplastin	Cell adhesion, activation of p38 MAPK, membrane protein	2.094	2.38
B4DL55	Laminin beta-3 chain	Laminin	2.084	2.395

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
P05362	Intercellular adhesion molecule 1 (ICAM-1)	Signalling receptor activity, integrin binding, membrane protein	2.063	2.217

Of the proteins which were downregulated in only the VECs infected with *C. albicans* alone or *C. albicans*+GBS (Table 5-5), eight are involved in apoptosis regulation. Several other proteins are associated with organisation of the cell cytoskeleton.

Table 5-5 VEC proteins relevant to infection which were downregulated both when VECs were incubated with *C. albicans* and *C. albicans*+GBS which were most relevant to infection

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q0P5N8	TMSB4X protein	Cytoskeletal organisation of actin, inhibits actin polymerisation	0.11	0.059
Q96FS1	CTNND1 protein	Cell adhesion, signal transduction	0.123	0.237
J3KP58	CAP-Gly domain-containing linker protein 1	Cytoskeletal organisation of microtubules	0.147	0.155
Q92736	Ryanodine receptor 2	Calcium channel, membrane protein	0.153	0.143
E7EMW0	Centrosomal protein of 170 kDa	Cytoskeletal organisation of microtubules	0.214	0.149
B4DSG5	Tax1-binding protein 1	Inhibits apoptosis, inflammatory response	0.26	0.219
Q9P0C6	HSPC255	Activates MAPK	0.31	0.339
Q15438	Cytohesin-1	Membrane transport, membrane protein	0.313	0.276
Q14680	Maternal embryonic leucine zipper kinase	Apoptosis, cell cycle regulation	0.331	0.247
O75676	Ribosomal protein S6 kinase alpha-4	Intracellular signalling, regulation of NF-kappa-B, inflammatory response	0.351	0.342
Q01546	Keratin, type II cytoskeletal 2 oral	Intermediate filament, cytoskeletal protein	0.352	0.322

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q6S5L8	SHC-transforming protein 4	Tyrosine kinase binding, may have a role in apoptosis, membrane protein	0.365	0.323
F8VW41	Phosphofurin acidic cluster sorting protein 2	Apoptosis, ion trafficking	0.366	0.35
STK17A	Serine/threonine kinase 17a (Apoptosis-inducing)	Induces apoptosis	0.376	0.429
Q7L3V2	Protein Bop	Induces apoptosis	0.401	0.486
P02533	Keratin, type I cytoskeletal 14	Intermediate filament, cytoskeletal protein	0.406	0.388
MYD88	Myeloid differentiation primary response protein MyD88	MyD88, inflammatory response, upregulation of cytokines and chemokines, NF-kappa-B activation	0.419	0.365
P26045	Tyrosine-protein phosphatase non-receptor type 3	Protein tyrosine phosphatase, membrane protein	0.42	0.375
A8K2I0	Keratin 6A	Intermediate filament, cytoskeletal protein	0.424	0.379
P35080	Profilin-2	Cytoskeletal organisation of actin, inhibits actin polymerisation	0.435	0.462
Q5VZL5	Zinc finger MYM-type protein 4	Cytoskeletal organisation	0.435	0.472
P15924	Desmoplakin	Desmosome organisation, membrane protein	0.46	0.437
H0Y7R8	Afadin	Signal transduction, cell adhesion, membrane protein	0.46	0.437
H0YAT7	Dystonin	Cytoskeletal linker protein	0.465	0.497
Q6LC01	Tubulin beta chain	Component of cytoskeleton (microtubules)	0.467	0.379
J3KPZ4	Nuclear nucleic acid-binding protein C1D	Apoptosis	0.475	0.455
P13647	Keratin, type II cytoskeletal 5	Intermediate filament, cytoskeletal protein	0.48	0.443

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q9BWT7	Caspase recruitment domain-containing protein 10	Apoptosis, activates NF-kappa-B	0.484	0.313

5.2.2.3 VEC response to dual-species infection

A total of 299 VEC proteins were downregulated in only the dual-species-infected VECs, and 136 were upregulated in only the dual-species-infected cells (see Appendix 7 and Appendix 8 for complete lists). When assigned to the five categories described earlier, 'biological process' comprised 14 categories (Figure 5-7A), 'cellular component' comprised 8 (Figure 5-7B), 'molecular function' comprised 7 (Figure 5-7C), 'protein class' comprised 22 (Figure 5-8) and 'pathway' comprised 75 (Figure 5-9). Among the 'biological process' category, the most significantly overrepresented terms comprised 'cellular process' and 'metabolic process'. This was seen for both up- and downregulated proteins, although the latter were most abundant (Figure 5-7A). A similar pattern was seen for category 'cellular component', for which the most significantly enriched terms were 'cell part', 'macromolecular complex' and 'organelle' (Figure 5-7B). Within the 'molecular function' category, downregulated proteins belonging to the terms 'catalytic activity' or 'binding' were the most abundant (Figure 5-7C).

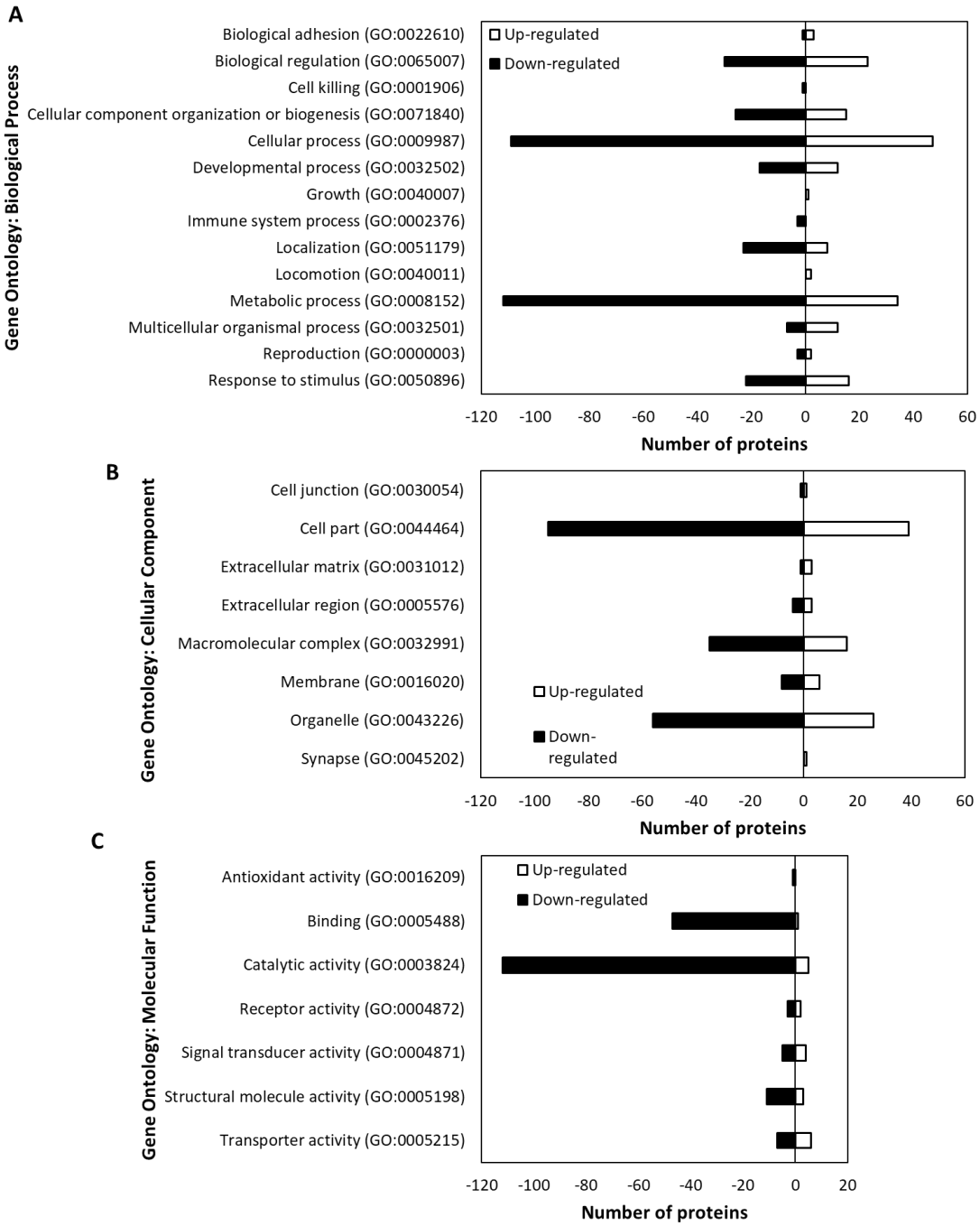

Figure 5-7 Distribution of VEC proteins grouped into GO categories that were significantly altered in dual-species infected cells.

Significantly upregulated proteins, white bars; significantly downregulated proteins, black bars. A) Biological process, B) cellular component, C) molecular function. Proteins included across all terms exhibited ≥2-fold expression difference relative to uninfected VECs; n=2.

Again, the most altered proteins within the category 'protein class' were downregulated (Figure 5-8). These proteins were assigned across a number of different terms, the top three of which were 'hydrolase', 'transferase' and 'oxidoreductase'. Finally, within the

category 'pathway', significantly expressed proteins were distributed across numerous terms (Figure 5-9). Once again, however, downregulated proteins were the most abundant. Of these, proteins associated with the term 'p53 pathways' were the most altered. For upregulated proteins, the most significant term was 'integrin signalling pathway'.

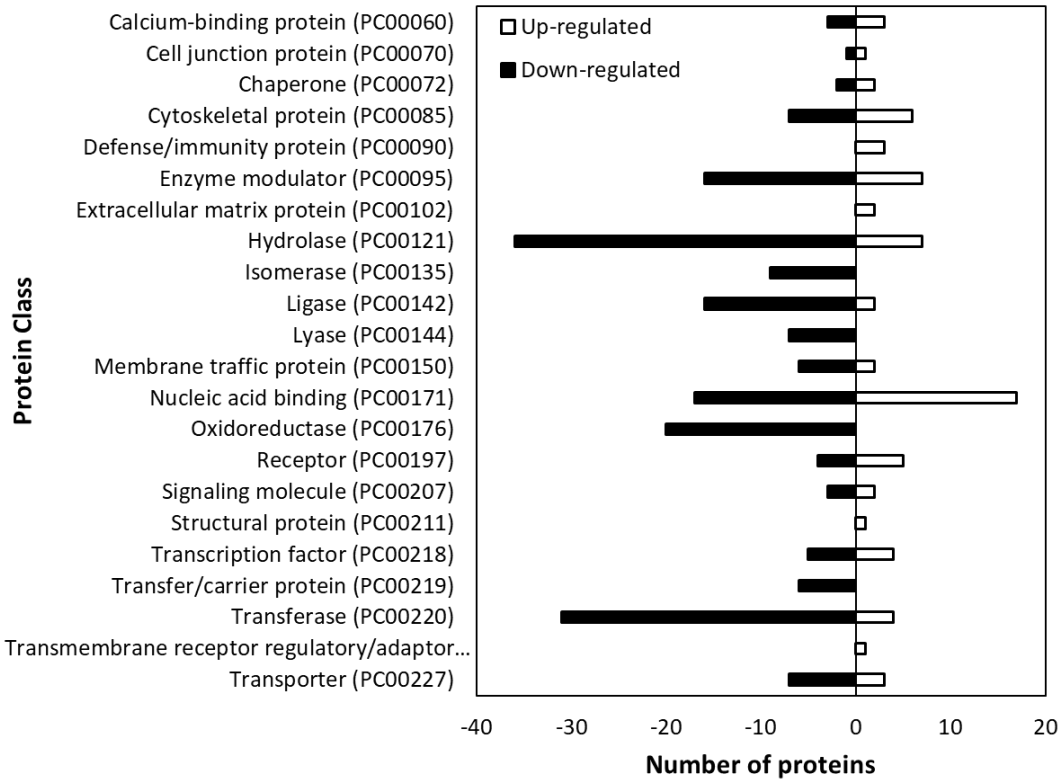

Figure 5-8 Distribution of VEC proteins grouped into category 'protein class' that were significantly altered in dual-species infected cells.

Significantly upregulated proteins, white bars; significantly downregulated proteins, black bars. Proteins included across all terms exhibited ≥2-fold expression difference relative to uninfected VECs; n=2.

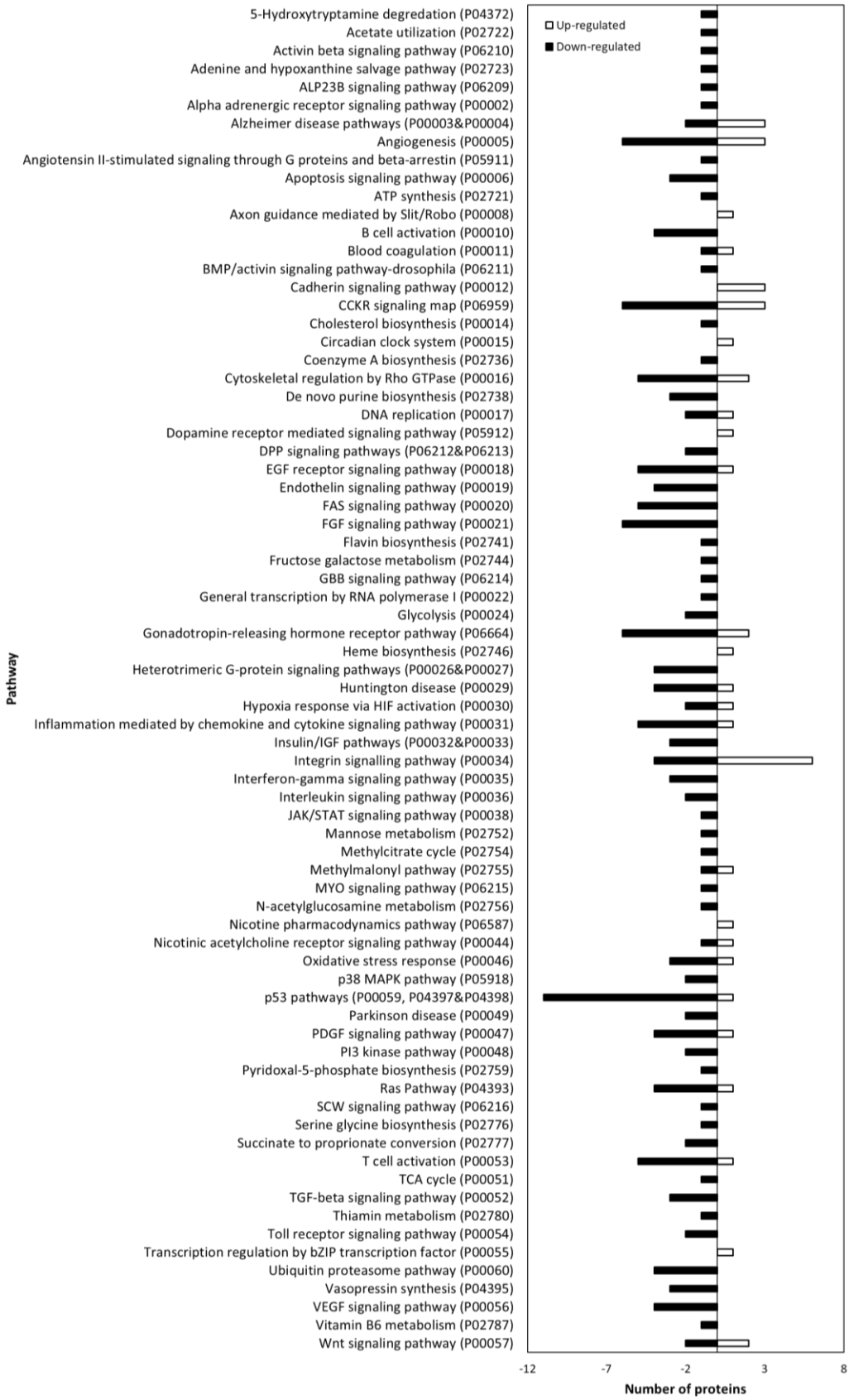

Figure 5-9 Distribution of VEC proteins grouped into category 'pathway' that were significantly altered in dual-species infected cells.

Significantly upregulated proteins, white bars; significantly downregulated proteins, black bars. Proteins included across all terms exhibited ≥2-fold expression difference relative to uninfected VECs; n=2.

As before, to build on the overview provided by the PAnThER analysis, individual proteins were also investigated. Of these selected proteins, those that were significantly upregulated are shown in Table 5-6. Among these VEC proteins, the most common theme was proteins associated with the actin cytoskeleton and its organisation. Proteins were also identified that may have capacity to serve as potential receptors for *C. albicans* and/or GBS. These included ECM proteins, such as laminin and collagen, which may act as bridging molecules, or surface-expressed determinants such as CD59. Regulatory proteins associated with immune or proinflammatory responses also exhibited enhanced expression. These included arginase-1 and four proteins that are associated with activation of NF-kappa-B. Arginase-1 has been shown to be upregulated in macrophages in response to chitin in the cell wall of *C. albicans*, preventing the induction of nitric oxide and thus reducing the antifungal activity of the macrophages (Wagener et al., 2017). In intestinal epithelial cells, excess arginase-2 production was linked to a reduction in nitric oxide production in response to *E. coli* LPS, as well as a reduced rate of apoptosis (Talavera et al., 2017). Upregulation of arginase-1 in VECs may play a similar role. CD99 antigen is a cell surface protein involved in the migration of leukocytes, adhesion of T-cells and death of T-cells by a caspase-independent pathway. Although CD99 is primarily expressed on the surface of haematopoetic cells such as lymphocytes (Petri and Bixel, 2006), more recently it has also been found to be expressed on epithelial cells (Krisanaprakornkit et al., 2013). Upregulation of this protein in the dual-species infection may signify increased immune activation in response to GBS and *C. albicans*.

Table 5-6 VEC proteins which were specifically upregulated in dual-species infections most relevant to human infection

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
H7C2F2	CD99 antigen	Cytoskeletal organisation of actin	4.059
Q86UE4	Protein LYRIC	Activates NF-kappa-B	3.585
F8WE98	Filamin-A	Cytoskeletal organisation of actin	3.351
H0Y2V6	Centrosomal protein of 170 kDa	Cytoskeletal organisation of microtubules	3.127
P05089	Arginase-1	Regulator of immune responses	3.09

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
P49711	Transcriptional repressor CTCF	MHC class II activator	2.83
A0A024R2V2	Microtubule-associated protein 4	Cytoskeletal organisation of microtubules	2.8
Q14686	Nuclear receptor coactivator 6	Activates NF-kappa-B	2.693
A0A0R9RWK2	Erb-b2 receptor tyrosine kinase 2	Binds EGF receptor to stabilise ligand binding	2.607
E9PR17	CD59 glycoprotein	Inhibits complement-mediated cell lysis	2.585
H7BZB9	Microtubule-associated protein 2	Cytoskeletal protein	2.547
Q9H270	Vacuolar protein sorting-associated protein 11 homolog	Intracellular trafficking, membrane bound	2.511
M0R0H3	mRNA decay activator protein ZFP36	Suppresses TNF-α	2.462
K7ENT6	Tropomyosin alpha-4 chain	Cytoskeletal organisation of actin	2.42
Q13443	Disintegrin and metalloproteinase domain-containing protein 9	Cytoskeletal organisation, mediates cell motility, cell-cell and cell-matrix interactions	2.393
P35222	Catenin beta-1	Transcriptional regulation, membrane bound, localises to adherens junctions and affects integrity of epithelial monolayer	2.319
V9GYF0	Rho guanine nucleotide exchange factor 2	Activates NF-kappa-B	2.276
G3XAI2	Laminin subunit beta-1	ECM protein	2.251
P28908	Tumor necrosis factor receptor superfamily member 8	TNF receptor, NF-kappa-B activation	2.242
Q06828	Fibromodulin	TGF-β receptor assembly	2.24
I3L0Y6	NmrA-like family domain-	Negatively regulates NF-kappa-B	2.236

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	containing protein 1		
P13645	Keratin, type I cytoskeletal 10	Cytoskeletal protein	2.232
P11047	Laminin subunit gamma-1	ECM protein	2.226
A7MBN3	Collagen, type IV, alpha 5	ECM protein	2.222
Q96SB3	Neurabin-2	Cytoskeletal organisation of actin	2.213
B4E0X1	Beta-2-microglobulin	MHC class I component, involved in antigen presentation	2.191
Q8N129	Protein canopy homolog 4	TLR4 regulation	2.155
P13726	Tissue factor	Involved in cytokine receptor activity, membrane bound	2.115
Q9BRA0	N-alpha-acetyltransferase 38, NatC auxiliary subunit	Apoptosis repressor	2.111
Q86YL7	Podoplanin	Cytoskeletal organisation	2.107
Q13092	Epidermal type I keratin	Cytoskeletal protein	2.095
P14316	Interferon regulatory factor 2	MHC class I repression, cell cycle regulation	2.091
Q92508	Piezo-type mechanosensitive ion channel component 1	Epithelial adhesion, membrane bound	2.089
P62745	Rho-related GTP-binding protein RhoB	Intracellular trafficking, membrane bound	2.077
O75712	Gap junction beta-3 protein	Gap junction, membrane bound	2.061
P81605	Dermcidin	Antimicrobial peptide	2.052
P25942	Tumor necrosis factor receptor superfamily member 5	Mediates signalling from members of TNF receptor superfamily	2.043
A0A0S2Z4G4	Tropomyosin 3	Cytoskeletal organisation of actin	2.039
C9JTA2	Mitotic spindle assembly	Cell cycle progression	2.022

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	checkpoint protein MAD1		
P08572	Collagen alpha-2(IV) chain	ECM protein	2.014

Of the downregulated VEC proteins (Table 5-7), eleven proteins were downregulated which are involved in cytoskeletal organisation. Seven proteins were involved in promotion of apoptosis, suggesting that the microbes may work to promote VEC survival and thus evade immune clearance. Likewise, several downregulated proteins are associated with the MAPK pathway, which plays a key role in the epithelial immune response to *C. albicans* and GBS. However, there were also proteins for which downregulation may be expected to result in the induction of an immune response. For example, ubiquitin thioesterase otulin limits proinflammatory cytokine activation and negatively regulates NF-kappa-B. Downregulation of a NF-kappa-B repressor correlates with the concomitant upregulation of several proteins that promote NF-kappa-B activation.

Table 5-7 VEC proteins which were specifically downregulated in dual-species infections most relevant to human infection

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
P14373	Zinc finger protein RFP	Ubiquitination, induces apoptosis	0.01
O76095	Protein JTB	Cell cycle progression, may inhibit apoptosis induced by TGFB1	0.273
P36542	ATP synthase subunit gamma	ATP Synthase, membrane bound	0.276
O95470	Sphingosine-1-phosphate lyase 1	Fatty acid enzyme, involved in apoptosis signalling	0.294
A8K710	Sterile alpha motif and leucine zipper containing kinase AZK	MAPK pathway, pro-apoptotic activity	0.353

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q96KB5	Lymphokine-activated killer T-cell-originated protein kinase	MAPK pathway	0.354
A0A140VK27	Leukotriene A(4) hydrolase	Degrades neutrophil chemoattractant	0.364
A8K3B6	Tyrosine-protein kinase	Suppresses TCR and BCR signalling	0.367
P67812	Signal peptidase complex catalytic subunit SEC11A	Signal transduction, membrane bound	0.38
L7RSM2	Mitogen-activated protein kinase	MAPK pathway, activated by proinflammatory cytokines	0.387
P61086	Ubiquitin-conjugating enzyme E2 K	Ubiquitin conjugating enzyme, involved in processing of NF-kappa-B	0.39
B3KY29	Poly [ADP-ribose] polymerase	Cell cycle progression	0.396
Q00013	55 kDa erythrocyte membrane protein	Signal transduction, membrane bound	0.396
B2R4D5	Actin-related protein 2/3 complex subunit 3	Cytoskeletal organisation of actin	0.399
A0A024QZN9	Voltage-dependent anion channel 2	Apoptosis induction	0.406
Q16539	Mitogen-activated protein kinase 14	MAPK pathway, activated by proinflammatory cytokines	0.409
A0A024R172	Leukotriene B4 12-hydroxydehydrogenase	Chemoattractant leukotriene B4 inactivation	0.414
P42574	Caspase-3	Activation of caspases responsible for apoptosis	0.415
Q53GP2	Thioredoxin-like 4B variant	Cell cycle progression	0.416
F8VZY9	Keratin, type I cytoskeletal 18	Cytoskeletal protein	0.429
O75381	Peroxisomal membrane protein PEX14	Intracellular protein transport, membrane bound	0.431
Q96BN8	Ubiquitin thioesterase otulin	Regulates innate immune response to	0.436

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
		limit proinflammatory signalling, negative regulation of NF-kappa-B	
A8K9G4	FLJ77745	Regulates TNF-α	0.439
Q9P287	BRCA2 and CDKN1A-interacting protein	Cytoskeletal organisation of microtubules	0.447
P68366	Tubulin alpha-4A chain	Cytoskeletal protein	0.456
Q9H4B6	Protein salvador homolog 1	Promotes apoptosis	0.459
A8K669	FLJ78452	Peptide processing for MHC class II	0.46
P55212	Caspase-6	Caspase, overexpression leads to apoptosis	0.46
B0I1P6	Kidney ankyrin repeat-containing protein 4	Cytoskeletal organisation of actin	0.462
P04843	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1	Catalytic enzyme, membrane bound	0.465
A0A024R687	Pleckstrin homology domain containing, family C (With FERM domain) member 1	Cytoskeletal organisation of actin, interacts with integrin	0.465
G3V5T9	Cyclin-dependent kinase 2	Cell cycle progression regulation	0.466
Q9BTV5	Fibronectin type III and SPRY domain-containing protein 1	Cytoskeletal organisation of microtubules	0.466
A0A024R275	Riboflavin kinase	TNF-α pathway	0.47
Q8TE77	Protein phosphatase slingshot homolog 3	Cytoskeletal organisation of actin filaments	0.471
Q02750	Dual specificity mitogen-activated protein kinase kinase 1	MAPK pathway	0.476
A0A0S2Z3H1	Caspase 2 apoptosis-related cysteine peptidase	Caspase	0.477
A0A0S2Z542	Mitogen-activated protein kinase	MAPK pathway, activated by	0.479

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
		proinflammatory cytokines	
Q06124	Tyrosine-protein phosphatase non-receptor type 11	Cell signalling, positively regulates MAPK signaling	0.48
A0A0G2JH58	MHC class I polypeptide-related sequence B	MHC class I pathway	0.481
F5H5D3	Tubulin alpha chain	Cytoskeletal protein	0.482
P07737	Profilin-1	Cytoskeletal organisation of actin	0.483
P27449	V-type proton ATPase 16 kDa proteolipid subunit	Proton transport, membrane bound	0.483
P10586	Receptor-type tyrosine-protein phosphatase F	Cell adhesion receptor	0.485
P55786	Puromycin-sensitive aminopeptidase	Antigen processing for MHC class I	0.49
O95999	B-cell lymphoma/leukemia 10	Adaptive immune response, promotes apoptosis and activation of NF-kappa-B	0.49
P05783	Keratin, type I cytoskeletal 18	Cytoskeletal protein	0.492
Q9NZN3	EH domain-containing protein 3	Cytoskeletal organisation of microtubules	0.492
Q9BRA2	Thioredoxin domain-containing protein 17	Modulates TNF-α signalling and NF-kappa-B activation	0.493
A0A087X1U6	Epiplakin	Cytoskeletal organisation of keratin	0.494
O60499	Syntaxin-10	Intracellular protein transport, membrane bound	0.499

5.2.3 Real-time RT-PCR studies

To complement the global analysis approach offered by the proteomics experiments (section 5.2.2), additional studies were performed to determine what effects the GBS-*C. albicans* interactions had on the VECs with regards to a proinflammatory cytokine response. RNA was harvested from VECs infected with GBS, *C. albicans* or both, and converted to cDNA. Real-time PCR was then performed using primer sets to identify the

relative expression levels of genes encoding six cytokines known to be associated with GBS or *C. albicans* disease: IL-1α, IL-1β, IL-8, IL-17, IL-23, and IL-36γ, with GAPDH used as the endogenous control (Patras et al., 2015a;Patras and Nizet, 2018;Mikamo et al., 2004;Doran et al., 2002). Real time RT-PCR was carried out on VEC samples infected with NEM316 or COH1. COH1 was used rather than 515 due to the neutrophil chemotaxis data, detailed in section 5.2.1, which showed that conditioned media from VECs infected with COH1 induced a greater neutrophil response, for which the centre of mass ‖ and FMI ‖ were comparable to that of the FMLP positive control (Figure 5-2).

In general, a more dramatic induction of cytokine transcript expression was detected with NEM316 than with COH1. Interestingly, a different profile was generated dependant on GBS strain. This may correlate with the enhanced cytotoxicity observed when VECs were incubated with NEM316 for an extended period (Chapter 3).

For NEM316 (Figure 5-10A), the transcript levels for IL-8, IL-17 and IL-36γ were significantly higher in the dual-species infected cells than the sum of the two monospecies infected cells, implying a possible synergistic effect with regards to expression of these genes. Transcript levels for IL-1α and IL-23 were comparable for the dual-species and *C. albicans* monospecies samples. No significant difference was seen in transcript levels for IL-1β across any of the samples. For COH1 (Figure 5-10B), significantly enhanced expression of the IL-8 gene was seen for the dual-species infection relative to the combined monospecies levels. Transcript levels for IL-1β, IL-17 and IL-23 were comparable for dual-species and *C. albicans* monospecies samples, and no significant differences were seen for IL-1α or IL-36γ across any of the samples.

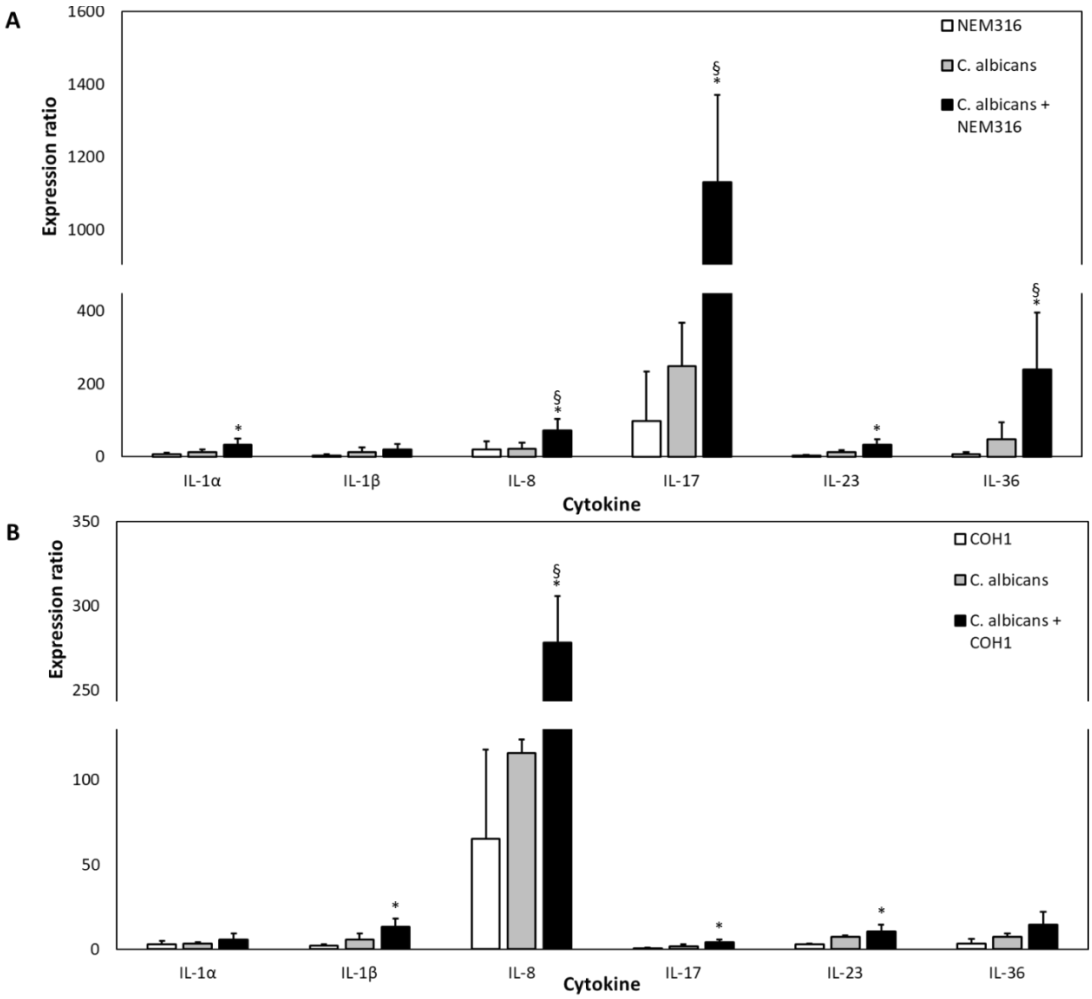

Figure 5-10 Expression ratios of cytokines produced by VECs in response to infection with GBS and/or *C. albicans*.

VECs were grown to confluence in 75 cm^2 tissue culture flasks before incubation with either *C. albicans* (MOI 2.5, grey bars) for 4 h or GBS (MOI 5, white bars) for 3 h. Alternatively, VECs were incubated with *C. albicans* for 1 h followed by addition of GBS and incubation for a further 3 h (black bars). Monolayers were disassociated, harvested and RNA was extracted using the Qiagen RNeasy Mini kit. Levels of cytokine transcripts were determined by real-time RT-PCR. A) VECs incubated with NEM316; n=4, B) VECs incubated with COH1; n=3. * indicates $P<0.05$ relative to VECs incubated with GBS, § indicates $P<0.05$ relative to VECs incubated with *C. albicans*, as established by one-way ANOVA with Tukey post-test.

5.3 Discussion

The data presented in this chapter sought to explore the host response to GBS-*C. albicans* interactions. To do this, the capacity of these microbes to generate a chemotactic neutrophil response was investigated, alongside their capacity to modify expression of chemotactic or proinflammatory cytokines. To gain a more global perspective, changes in proteome profiles of the VECs were examined also.

5.3.1 Neutrophil chemotaxis

Neutrophil activation was considered for these studies as neutrophils are the most abundant immune cell in the vaginal tract (Nandi and Allison, 1993). When neutrophils are more activated in terms of motility, they are more likely to eradicate infection (Mantovani et al., 2011). Furthermore, there was precedent for bacterial interactions with *C. albicans* to modulate neutrophil behaviour. Peters and Noverr (2013) described a *C. albicans-S. aureus* coinfection that resulted in upregulation of proinflammatory cytokines that, in turn, altered the neutrophil response, leading to enhanced virulence (Peters and Noverr, 2013). As might be predicted, the data from the studies presented here indicated that neutrophils showed more activity when exposed to microbial-infected conditioned media than VEC-only conditioned media. This confirmed that the VECs were not secreting chemokines without microbial stimulation. Moreover, it was found that neutrophils were more active and exhibited greater directionality (i.e. neutrophils were aware of the location of their target) when cells were infected with GBS alone rather than when cells were infected with *C. albicans* followed by GBS. This may indicate that *C. albicans* physically impairs GBS from mediating its effects, effectively masking the presence of GBS. Alternatively, prior incubation with *C. albicans* could initiate a response in the VECs that predominates, even following GBS exposure. It is well established that GBS has capacity to induce neutrophil recruitment via toxin β-H/C, which induces expression of a number of neutrophil chemoattractants. This has been demonstrated using HBMEC monolayers *in vitro* (Doran et al., 2003), and using animal models (Doran et al., 2002;Hensler et al., 2005). This infers that the latter mechanism may be more likely. Regardless of the mechanism, diminished neutrophil recruitment upon coinfection might be expected to promote colonisation of the GU tract by these microbes and possibly promote microbial dissemination. However,

activation of neutrophils and subsequent infiltration into host tissues does not necessarily mean that the immune response will successfully clear an infection and indeed, reduced neutrophil recruitment may serve to dampen a symptomatic host response to coinfection. This is because it has been implied that neutrophils in the vaginal tract may play an aggravating rather than protective role (Fidel et al., 2004), leading to an exacerbated inflammatory response and deteriorating pathology (Yano et al., 2014;Yano et al., 2012).

From these studies, no strong chemotactic effect was seen for VECs incubated with *C. albicans* alone. This differs from previous work, in which culture filtrate of *C. albicans* has been found to contain chemoattractants for both neutrophil and macrophage cells (Edens et al., 1999). The neutrophil chemoattractant was effective across an intestinal epithelial cell line and was found to act via the formyl peptide receptor of neutrophils (Edens et al., 1999). This observation was also supported by (Brasch et al., 1992;Cutler, 1977), who detected the presence of the chemotactic factor and showed that *C. albicans* attracted neutrophils. A further study estimated that the *C. albicans* neutrophil chemoattractant was 1 kDa in size, and not expressed by opaque-phase cells (Geiger et al., 2004). Autophagy by VECs, triggered following invasion by *C. albicans*, is another mechanism that has been shown to upregulate release of proinflammatory cytokines, leading to neutrophil recruitment (Shroff et al., 2017;Shroff et al., 2018). Such differences between our findings and those of these previous studies likely reflect variations in the experimental parameters e.g. *in vitro* vs. *in vivo* models, and different MOI, incubation periods and *C. albicans* strains. To try and explore the potential for strain-specific effects, it could be beneficial to repeat the studies performed here with a wider panel of *C. albicans* strains.

One of the potential drawbacks to these assays is that centrifugation of the conditioned media is unlikely to remove all microbes or microbial by-products, which may then affect the outcome of these assays. However, across the parameters tested, neutrophils showed less chemotaxis when exposed to conditioned media from dual-species infected VECs when compared to monospecies-infected VECs. Since GBS interactions with VECs were promoted by the presence of *C. albicans*, and vice-versa, VECs infected with both GBS and *C. albicans* were exposed to significantly higher numbers of microbes than when incubated with the single species. If the neutrophils were responding solely to microbes or microbial by-products which had failed to be removed from the

supernatant, it could be expected that a much stronger signal would be observed in response to the dual-species rather than monospecies conditioned media. As this was not the case, it is unlikely that these potential confounding factors would have significantly affected the results shown here. Furthermore, centrifugation to remove microorganisms is an established method (Hill et al., 1988).

Further possible limitations of the neutrophil chemotaxis data include sampling bias. Neutrophils were excluded from the dataset if they could not be tracked for the full 31 time points. When neutrophils collide or cross paths, Fiji cannot decipher which neutrophil is which so the tracks are cut short. This means that neutrophils which are stationary are more likely to be included, while neutrophils which are very active or moving quickly are more likely to be excluded. Furthermore, neutrophils which pass outside of the field of view cannot be tracked for the full number of time points. However, if anything, such effects would likely have underrepresented the chemotactic effects of the microbes rather than skewing the data interpretation. A further issue was that the stage of the microscope occasionally shifted over the course of the 1 h, meaning that tracks had to be manually adjusted to account for this. To do this, stationary artefacts on the chemotaxis slide were tracked, and the average movement was subtracted from test tracks. However, again, this adjustment should not have affected the overall data interpretation.

5.3.2 Proteomics

Significant differences were seen in the number of modulated VEC proteins across the different infection scenarios. GBS NEM316 seemed to have least impact on the VEC proteome, while *C. albicans* was much more stimulatory. Both microbes are known to be cytotoxic and microarray profiling has shown a strong response of VECs to GBS at the transcriptional level (Patras et al., 2013). The differences observed here may therefore reflect temporal variation in the effects mediated by these two microbes and thus a different outcome may be observed using a longer incubation period, for example. Nonetheless, from these studies it was clear that coinfection by *C. albicans* and GBS had a significant impact on the VEC response, above that seen with the monospecies infections.

While challenging to predict exactly which of the modulated proteins might significantly impact the nature of VEC interactions with *C. albicans* and GBS, some common themes were seen in the functions of the affected proteins in response to the dual-species infection. One such theme was organisation of the cytoskeleton. The cytoskeleton is known to be a target for both GBS and *C. albicans*, with induction of cytoskeletal rearrangements promoting invasion of epithelial tissues and possible dissemination to distant sites (Dalle et al., 2010). Invasion of GBS into A549 and HEp-2 epithelial cells was shown to be dependent on actin filaments (Rubens et al., 1992;Valentin-Weigand et al., 1997;Baron et al., 2004), involved activation of unidentified protein kinase signalling pathways, and was dependent on extracellular calcium concentrations (Valentin-Weigand et al., 1997). *C. albicans* can invade host cells by two mechanisms: induced endocytosis, mediated by hyphae surface proteins Als3 and Ssa1, and active penetration, which is less well characterised (Mayer et al., 2013;Sun et al., 2010;Phan et al., 2007). Induced endocytosis may occur when fungal cells are non-viable. However, active penetration is initiated by fungal cells themselves. Lytic enzymes such as SAPs and the physical pressure of hyphae growth are thought to contribute to active penetration, although the specific proteins which mediate this are, as of yet, unknown (Wachtler et al., 2011;Felk et al., 2002;Naglik et al., 2003;Gow et al., 2002). Both Als3 and Ssa1 bind cadherins and are thought to induce endocytosis in a clathrin-dependent mechanism (Phan et al., 2007;Sun et al., 2010). However, another study has suggested that induced endocytosis may be due to macropinocytosis, where extracellular material is non-selectively engulfed by membrane ruffles (Dalle et al., 2010). Similar to GBS, induced endocytosis by *C. albicans* filaments is known to be actin-dependent and involves reorganisation of the cytoskeleton (Yang et al., 2014). The fact that a number of VEC proteins which regulate actin were altered in expression suggests that *C. albicans*-GBS coinfection may enhance the invasive potential of these microbes.

Moreover, modulation of host cytoskeletal proteins has implications for the integrity of the epithelial barrier. Widely studied for *E. coli* and *Salmonella* spp., actin modulation can enable bacterial disruption of the epithelium, leading to invasion and dissemination within the host (Navarro-Garcia et al., 2013;Van Nhieu and Romero, 2017;Zhou et al., 2001). Further to this, GBS has been described to utilise beta-catenin and integrin signalling to enable ascending infection of the vaginal tract (Vornhagen et al., 2018). The enhancement of upregulated proteins involved in integrin signalling and beta-catenin itself in dual-species-infected VECs could suggest a role for the synergistic relationship

between *C. albicans* and GBS to promote ascending GBS uterine infection and epithelial exfoliation, which may be another mechanism by which epithelial integrity is compromised by GBS, although this would have to be examined in more detail in further studies.

Another common function amongst the modulated VEC proteins was activation of regulator NF-kappa-B. This is interesting, as the epithelial immune response to *C. albicans* is triggered in part following recognition of yeast via NF-kappa-B and MAPK responses, leading to downstream activation of cytokines and chemokines (Naglik et al., 2014). This suggests a possible increase in downstream proinflammatory signalling, which would correlate with the real-time RT-PCR cytokine data. NF-kappa-B is activated in response to *C. albicans* in both oral and vaginal epithelial cells. However, different downstream signalling pathways appear to be activated, as a different panel of cytokines is produced by oral epithelial cells than is produced by vaginal epithelial cells (Moyes et al., 2011). GBS also activates NF-kappa-B (Vallejo et al., 2000). GBS interaction with TLRs on the surface of host cells leads to activation of NF-kappa-B and subsequent downstream signalling, and this was partially dependent on MyD88 expression (Henneke et al., 2002). Activation of NF-kappa-B by GBS in endothelial cells has also been shown to induce nitrogen oxide synthase (Glibetic et al., 1999). Furthermore, in monocytes GBS was shown to activate MAPKs and NF-kappa-B in order to upregulate expression of TNF-α, a proinflammatory cytokine linked to enhanced pathology of GBS disease such as septic shock and increased risk of death (Mancuso et al., 2002). The data presented here suggest that dual-species infection may be enhancing VEC proinflammatory response in a synergistic manner.

Both GBS and *C. albicans* have been described to bind a wide range of host components, such as ECM proteins. The data here showed upregulation of ECM proteins laminin and collagen. GBS surface proteins which bind these ECM components include laminin-binding protein Lmb (Tenenbaum et al., 2007), laminin-binding protein BsaB (Jiang and Wessels, 2014), and Bsp proteins, which bind both collagen and laminin (Brady et al., 2010). *C. albicans* also binds these ECM components (Yan et al., 1998). The Tdh1 adhesin of *C. albicans* has been shown to bind laminin (Villamon et al., 1999), while Als3 has been shown to bind collagen, as this ability was conferred upon a *S. cerevisiae* strain heterologously expressing Als3 (Bamford et al., 2015). *C. albicans* hyphal proteins Ssa1 and Als3 also specifically target cadherins, components of adherens junctions between

host cells (Sun et al., 2010;Phan et al., 2007). As such, upregulation in dual-species infected VECs of 2 laminin subunits and 2 collagen proteins, as well as modulation in expression of proteins involved in cytoskeletal modulation, is likely to affect the ability of GBS and *C. albicans* to interact with these host cells.

Several VEC proteins relating to regulation of apoptosis were significantly downregulated in dual-species samples. This suggests that *C. albicans* and GBS may promote host cell survival to further microbial persistence. Additionally, as seen for other cell types, upregulation of arginase-1 may lead to an inhibition in nitric oxide production, decreasing the antifungal activity of VECs, as well as potentially further decreasing the likelihood of apoptosis (Wagener et al., 2017;Talavera et al., 2017). Moreover, the downregulation in the p53 pathway described by the GO analysis could suggest dampening of the host stress response. p53 mediates repair of DNA, arrest of the cell-cycle leading to senescence, and apoptosis, indicating an additional mechanism by which GBS and *C. albicans* may work together to promote their survival (Vazquez et al., 2008).

Despite differences in the specific proteins, there was significant overlap in the ontology profiles and general functions of the affected proteins obtained from VECs exposed to *C. albicans* alone or both microbes together. This provides further evidence to support the notion that the VEC response to *C. albicans* may predominate and indeed, it seems as if the dual-species infection may actually serve to enhance this response.

5.3.3 Proinflammatory cytokine transcription

Proinflammatory cytokines are well documented as an initial host response to microbial infection. IL-1α, IL-1β, IL-17 and IL-23 were significantly increased in expression in a murine model of *C. albicans* vaginal infection when compared against control mice (Shroff et al., 2018), while IL-1α, IL-1β, IL-8, IL-23 and IL-36γ expression was induced by incubation of VECs with GBS strains A909 or COH1 (Patras et al., 2013). These cytokines were therefore the focus of the studies performed here to investigate the potential proinflammatory effects of GBS-*C. albicans* interactions on VECs.

In the studies performed here, there was a general trend for the transcription levels of all six cytokines by VECs to be higher from dual-species rather than monospecies infections. These effects appeared largely to be additive, implying that both *C. albicans* and GBS were inducing transcription of these cytokines independently, and for IL-23, the effects seemed to be predominantly driven by *C. albicans*. However, a synergistic effect was seen with IL-8 transcripts when VECs were challenged with either GBS strain. Additionally, a synergistic effect was seen for VECs infected with NEM316 and *C. albicans* with regards to transcription levels for IL-17 and IL-36γ.

IL-8 has been found to have strong chemoattractant activity, which is dose-dependent (Doran et al., 2002;Leonard et al., 1991), and facilitates the ability of neutrophils to kill some microbial pathogens, such as *E. coli*, *Mycobacterium fortuitum*, *P. aeruginosa* and *C. albicans* (Standiford et al., 1996;Nibbering et al., 1993;Ponglertnapagorn et al., 1996;Djeu et al., 1990). IL-8 has also been shown to increase expression of adhesion proteins on the surface of neutrophils (Detmers et al., 1990), enhance the ability of neutrophils to migrate through tissues (Huber et al., 1991), and to induce release of ROS and lysosomal enzymes (Peveri et al., 1988). IL-36γ is a member of the IL-1 cytokine family. It binds the IL-36 receptor to initiate similar cellular signalling events as IL-1, inducing proinflammatory cytokine release from active CD4+ T cells and dendritic cells (Vigne et al., 2011). IL-36γ also upregulates expression of MHC class II and CD80/86 to induce dendritic cell maturation (Vigne et al., 2011), and is able to induce MAPK and NF-kappa-B activation, leading to downstream activation of a number of genes, including the chemoattractant IL-8 (Towne et al., 2004). The significant induction of IL-36γ and IL-8 transcription by VECs following dual-species versus monospecies infection implies that the dual-species infection could have a much greater proinflammatory effect *in vivo*, with significant neutrophil recruitment. It might have been expected then that the dual-species conditioned media would have exhibited a greater neutrophil chemotactic effect than the conditioned media from the monospecies infections, which was not the case. Nonetheless, this may reflect the fact that the transcriptional effects at 4 h post-infection had yet to be translated into secreted protein levels. Consequently, collecting conditioned media from the association assays after a longer time frame might reveal different responses from the neutrophil chemotaxis assays. Invasion of host tissues has been suggested as the trigger for induction of IL-8, as IL-8 was detected upon entry of a range of bacteria into intestinal or cervical epithelial cells (Eckmann et al., 1993;Eckmann et al., 1995). The elevated transcription of IL-8 by VECs following dual-

species infection may therefore indicate a higher level of VEC internalisation by *C. albicans* and GBS when incubated together rather than independently. This supports data presented in Chapter 3. Candidalysin triggers p38-MAPK/c-Fos, NF-kappa-B, and PI3K signalling in oral and vaginal epithelial cells, leading to the expression of proinflammatory cytokines, including IL-36, IL-1α, and IL-1β (Verma et al., 2018;Richardson et al., 2017). It is possible, therefore, that the RT-PCR data are also indicative of a higher level of candidalysin production in dual-species infections compared to *C. albicans* alone. If true, this, in turn, could have implications for levels of potential tissue disruption with the GU tract following co-colonisation by *C. albicans* and GBS.

IL-17 may activate secretion of antimicrobial peptides and inflammatory mediators from neutrophils to control *C. albicans* infection (Eyerich et al., 2008) and has been described as crucial for *C. albicans* defence, although the involvement of IL-17 in VVC has little, and contrary, evidence (Sparber and LeibundGut-Landmann, 2015). The presence of IL-17 in the vaginal tract of mice has also been linked with clearance of GBS, and mice treated with recombinant IL-17 had significantly fewer colonising GBS (Patras et al., 2015a). Since dual-species infection of VECs with *C. albicans*/NEM316 resulted in greater IL-17 transcription than monospecies-infected cells, enhanced clearance of *C. albicans* and GBS from the vaginal tract might be anticipated following coinfection. Again, however, the issue of transcription versus translation is important, particularly as Th17 cells are known as the predominant source of IL-17. Nonetheless, there is evidence that IL-17 can be produced from epithelial cells, albeit from a study focused on the oral cavity (Konermann et al., 2013).

Taken together, these real-time RT-PCR data support prior studies by indicating that both GBS and *C. albicans* have capacity to induce expression of proinflammatory cytokines from VECs. Furthermore, there is evidence that coinfection by these microbes may elevate this response. Based on the varying cytokine profiles obtained from GBS strains NEM316 and COH1, it seems likely that the effects of GBS are strain-specific and to fully interpret the impact of these transcriptional responses, it would be important to also monitor levels of released protein over time. A useful next step with this work would therefore be to investigate a wider range of strains over a longer time period, and to additionally monitor levels of cytokine released into the culture medium, for example by ELISA or FACS. The innate immune response to GBS is also characterised by cytokines

such as TNF-α, IFN-β and IL-12, which are produced by cell types other than epithelial cells (Mancuso et al., 1997;Teti et al., 1993;Mancuso et al., 2007). Investigations into the effects of coinfection on these cytokines would be another area of interest for future work.

5.3.4 Summary

Taken together, these data highlight the complexity of the interactions between *C. albicans*, GBS and VECs. Nonetheless there was clear evidence that coinfection of VECs by these microbes did modulate the VEC response in a manner that correlates with their synergistic partnership. Under the conditions tested, this resulted in an apparent enhancement of the effects largely mediated by *C. albicans* alone, and might be predicted to promote microbial association with the epithelium and subsequent persistence. Together, these microbes demonstrated a capacity to promote the expression of proinflammatory cytokines at the transcriptional level, but chemotactic effects on neutrophils appeared to be suppressed.

Chapter 6 Discussion

GBS is the leading cause of neonatal sepsis and meningitis in the developed world, and is additionally thought to cause significant morbidity and mortality in neonates in the developing world (Edmond et al., 2012;Le Doare and Heath, 2013). In many cases, these diseases arise as a result of vertical transmission from a colonised mother. GBS can also cause infections in the elderly and in animals, particularly cattle and fish (Edwards and Baker, 2005;Nair et al., 2005;Evans et al., 2009). *C. albicans* causes a wide range of infections, mostly among immunocompromised individuals, and is often acquired nosocomially (Pfaller and Diekema, 2007;Mayer et al., 2013). *C. albicans* is also the most common causative agent of VVC, with particular incidence among pregnant women (Sobel, 2007). GBS and *C. albicans* occupy the same biological niche within the GU tract, and there are reports of synergism between *C. albicans* and related streptococci within the oral cavity (Bamford et al., 2009;Hwang et al., 2017;Diaz et al., 2012). Due to the disease burden caused by these two opportunistic pathogens, it was considered beneficial to investigate whether there was a similar synergism between *C. albicans* and GBS within the GU tract, and what the molecular basis for such an interaction could be. Such information could inform the development of future therapeutic interventions. These studies were therefore conceived to establish whether GBS facilitates *C. albicans* colonisation of VECs and vice versa, with additional aims of defining the molecular basis of any such interactions, and to investigate the effect of GBS-*C. albicans* interactions on the host cells.

6.1 Microbial interactions with VECs

6.1.1 GBS interactions with VECs

A key risk factor for neonatal GBS disease is vaginal colonisation of the mother (Baker and Barrett, 1974). GBS is known to bind VECs (Sheen et al., 2011), but the abilities of the specific GBS strains used here (Table 3-1) to interact with VECs clearly demonstrate that this process is strain-specific. Previous studies have inferred that GBS strains with CPS III exhibit higher levels of binding to VECs than CPS Ia (Bodaszewska-Lubas et al., 2013). This appears to be confirmed here, with NEM316 and COH1 (both CPS III) adhering to VECs at higher levels than 515 (CPS Ia). Nonetheless, this attribute alone

does not seem to dictate disease risk, since CPS types Ia, II, III and V are associated with the majority of GBS disease (Zhang et al., 2006;Jiang et al., 2008;Farley, 2001) and CPS Ia, III and V predominantly with neonatal disease (Alkuwaity et al., 2012;Weisner et al., 2004;Zaleznik et al., 2000).

In addition to expressing CPS III, GBS strain COH1 belongs to CC-17, which is particularly associated with neonatal disease and meningitis (Bohnsack et al., 2008;Martins et al., 2007;Martins et al., 2017;Poyart et al., 2008;Tazi et al., 2010). This has been linked to expression of PI-1 and PI-2b. Evidence suggests that PI-1 and PI-2a are expressed in most GBS strains, regardless of CPS type, while PI-2b is confined almost exclusively to CC-17 strains (Dramsi et al., 2012;Springman et al., 2014). Pili are involved in adhesion to host surfaces and biofilm formation (Dramsi et al., 2006;Konto-Ghiorghi et al., 2009;Maisey et al., 2007), and one study found that GBS isolated from invasive neonatal infections more frequently had both PI-1 and PI-2b than did colonising GBS strains from mothers. This suggested that, together, these two PI may enhance pathogenicity (Springman et al., 2014). Additionally, CC-17 GBS strains bind fibrinogen at higher levels than non-CC-17 strains (Dramsi et al., 2012), and this has been hypothesised to contribute to the elevated pathogenicity observed with these strains (Al Safadi et al., 2011). From these studies, it might have been anticipated that COH1 would be more invasive than the other GBS strains used and indeed, COH1 supernatant induced a greater level of neutrophil chemotaxis than NEM316, regardless of the presence of *C. albicans*. Since neutrophil infiltration has been associated with host tissue damage and pathogen dissemination (Yano et al., 2010;Fidel et al., 2004), it is possible that such effects may contribute to the enhanced pathogenicity reported for COH1.

6.1.2 GBS interactions with *C. albicans* and VECs

All five GBS strains interacted to some degree with *C. albicans* planktonically. Interactions were much less common and involved fewer bacteria for GBS strains 18RS21, 2603V/R and COH1, but coassociation was clearly seen between *C. albicans* and COH1 in the presence of VECs. This implies a common ability for GBS to interact with *C. albicans.* In all cases, GBS preferentially bound to *C. albicans* hyphae rather than blastospores. This echoes previous work with *S. gordonii* and *S. aureus*, both of which exhibit a tropism for *C. albicans* hyphae (Peters et al., 2010;Silverman et al., 2010).

Supporting the tropism of GBS for *C. albicans* hyphae observed during planktonic interactions, *C. albicans* was found to promote GBS in association with VECs only when in the filamentous morphology.

Strains 18RS21 (CPS II) and 2603V/R (CPS V) were only used in the initial studies in Chapter 3 and while able to associate with the VECs to high levels, these two strains did not aggregate strongly with *C. albicans*. This is potentially of note, as both of these strains belong to CC-19 that, along with CC-1 and -23, are more frequently isolated from asymptomatic maternal carriage than from neonatal disease (Jones et al., 2003;Luan et al., 2005;Manning et al., 2008). It would be interesting to determine if this was a trend seen across other strains belonging to CC-19, as this might support coassociation between GBS and *C. albicans* as a factor that can promote pathogenicity.

For all three GBS strains tested it was evident that the presence of *C. albicans* promoted numbers of GBS able to associate with VECs. Clearly, coadhesion between GBS and *C. albicans* was one mechanism by which this effect was mediated. Nonetheless, proteomics data suggested that the presence of both microbes may also directly modulate VECs in a manner that could make them more permissive to infection. For example, several AgI/II family proteins are known to bind ECM components such as laminin and collagen (Brady et al., 2010;Zhang et al., 2006;Rego et al., 2016b), both of which were upregulated in VECs following coinfection. It seems feasible, therefore, that *C. albicans* may serve as a bridging microorganism between GBS and VECs, as well as functioning alongside GBS to upregulate VEC receptors. Again, this would correlate with the pattern of VEC association seen with GBS upon coinfection, with bacteria bound both to *C. albicans* hyphae but also directly to the VEC monolayer. Coassociation between *C. albicans* and *S. gordonii* enhances the ability of these microbes to adhere to host surfaces and persist (Silverman et al., 2010). Likewise, interaction of *C. albicans* with *S. mutans* enables formation of multispecies biofilms, leading to a greater burden of both species orally (Hwang et al., 2017), while the burden of *S. oralis* within oral biofilms was dramatically increased by coinfection with *C. albicans* (Diaz et al., 2012). It might be anticipated then that association with *C. albicans* could enhance the burden of GBS within the GU tract, and may well represent a strategy that GBS, similar to other *Streptococcus* bacteria, has evolved to promote its colonisation and persistence within this ecological niche.

Internalisation into VECs by NEM316 and COH1 was promoted by *C. albicans* but this was not seen for strain 515. This correlated with the relative number of hypha-GBS interactions observed by confocal microscopy for these strains and suggests that, at least in part, the enhanced invasion by NEM316 and COH1 could have been due to 'piggybacking' as *C. albicans* hyphae penetrated the VECs. It is unclear why the presence of *C. albicans* was apparently inhibitory to 515 internalisation. However, if 515 preferentially invades via direct binding to VECs rather than *C. albicans* hyphae, it is possible that the extent of hyphal mat formation occluded the host cell receptors exploited by 515. Tissue invasion by 'piggybacking' on *C. albicans* hyphae is a mechanism utilised by *S. aureus* and was shown to result in enhanced bacterial dissemination and systemic disease (Schlecht et al., 2015b). It is interesting to note then that both NEM316 and COH1 bear CPS III, the serotype that is particularly associated with invasive disease, while 515 belongs to CC-23, one of the CC associated with asymptomatic carriage within the GU tract. Again, this might support a potential role for coassociation between GBS and *C. albicans* in contributing to the pathogenic profile of these microbes. Relating to this, cytotoxicity levels were significantly higher in VECs exposed to *C. albicans* and GBS than in VECs exposed to monospecies GBS or *C. albicans*, and may be due to the enhancement of GBS invasion by the presence of *C. albicans*. This was supported by proteomics investigations, which showed modulation of a number of VEC proteins involved in cell stress and these were detected at the highest levels for coinfections. Proteomic analysis of coinfections also demonstrated the modulation in expression of no fewer than 8 proteins relating to actin organisation. Given the fact that actin filaments coordinate invasion of both GBS and *C. albicans* into host cells (Rubens et al., 1992;Baron et al., 2004;Yang et al., 2014), these data suggest a greater invasive potential in the presence of both microbes. Again, this correlates well with the internalisation data for GBS in the presence of *C. albicans*. It is not possible to define from these studies if the enhanced VEC invasion directly related to the elevated levels of biomass for the two species, with both microbes inducing independent pathways, or if a synergistic effect was also occurring at the level of VEC signalling. Nonetheless, the fact that the modulation of several VEC proteins was unique to the coinfection, may provide evidence for the latter.

One example of VEC responses that were unique to coinfection, and with potential implications particularly to GBS disease risk, was upregulation of proteins related to integrin signalling. Integrin signalling is utilised by GBS alpha C protein to facilitate

internalisation (Bolduc and Madoff, 2007), and has also been linked to *S. gordonii* AgI/II family proteins SspA/B. Specifically, SspA/B promote *S. gordonii* interactions with HEp-2 epithelial cells via recognition of β_1 integrin (Nobbs et al., 2007). Thus, this VEC response correlates with enhanced GBS internalisation upon coinfection, and it would be of interest to determine if the Bsp proteins have a specific role in this process. Moreover, activation of integrin signalling has been shown to promote ascending GBS infection (Vornhagen et al., 2018). Consequently, upregulation of integrin signalling in dual-species-infected VECs may indicate that coinfection with *C. albicans* has capacity to enhance GBS ascending infection. This has implications for the pathology of GBS infections when *C. albicans* is present.

6.1.3 *C. albicans* interactions with GBS and VECs

Interactions between GBS and *C. albicans* were reciprocal and thus, *C. albicans* was significantly promoted in association with VECs when in the presence of GBS. However, *C. albicans* was promoted to the same level for each of the three GBS strains. Since the explicit numbers of GBS that associated with VECs were strain-dependent and thus variable, this may indicate an indirect effect(s) rather than as a consequence of direct physical interactions between the two microbes. This notion is supported by previous work showing promotion of *C. albicans* association with host cells by oral streptococci. *S. gordonii* promotes filamentation and thus biofilm formation in *C. albicans* through secretion of nutrients and hydrogen peroxide (Bamford et al., 2009;Nasution et al., 2008). Additionally, the effects on *C. albicans* of quorum sensing molecule farnesol, which negatively regulates hyphae formation, were reversed by co-incubation with *S. gordonii*, and this was due to suppression of farnesol by *S. gordonii* autoinducer-2 (Bamford et al., 2009). Likewise, *S. oralis* can enhance formation of *C. albicans* hyphae, leading to an increase in candidal tissue adherence and invasion (Diaz et al., 2012;Xu et al., 2017). Studies using transwell plates or conditioned media gave no evidence of secreted components from GBS being able to modulate *C. albicans* interactions with VECs. It cannot be ruled out, however, that in these studies diffusion distances or stability of the secreted component(s) were issues, and it may be a combined mechanism involving physical proximity and extracellular signals that enables GBS to promote *C. albicans* association with VECs. This could be explored further through use of, for example, GBS strains deficient in quorum sensing molecules such as *luxS*.

Alongside physical interactions and extracellular signalling, it is also possible that enhanced *C. albicans* association occurred as a consequence of changes in VEC protein expression induced following coinfection. As for GBS, *C. albicans* may benefit from elevated expression of ECM components collagen and laminin, which can be bound by a number of candidal receptors, including Als3 (Bamford et al., 2015;Villamon et al., 1999). Regardless of the precise mechanism(s) involved, it seems quite possible that *C. albicans* and GBS have co-evolved to exploit the presence of the other for colonisation and survival within the GU tract.

6.2 Molecular basis of microbial interactions

6.2.1 Role of AgI/II family proteins

AgI/II family proteins are expressed on the surface of many species within the *Streptococcus* genus. Among the oral streptococci, these proteins have been shown to act as major colonisation determinants, playing a role in binding to host surfaces and to members of the resident microbiota (Nobbs et al., 2009). The Bsp proteins of GBS are relatively small (<932 amino acid residues) in comparison to AgI/II family proteins of oral streptococci (1310-1653 amino acid residues) (Brady et al., 2010). Nonetheless, the data presented here, which indicates that BspA and BspC proteins can mediate interactions with VECs and *C. albicans*, add to a growing body of evidence that Bsp proteins may be similarly important in facilitating GBS colonisation of the GU tract.

6.2.1.1 Interactions with VECs

Complementing previous work on BspA by (Rego et al., 2016a), *bspC* knockout and complemented strains of GBS 515 and COH1 have been used here to demonstrate a role for BspC expression in VEC binding and internalisation. Furthermore, anti-Bsp antibodies impaired the ability of GBS NEM316 and 515 to interact with VECs, while *L. lactis* strains expressing BspA or BspC were enhanced in both VEC association and invasion compared to the *L. lactis* vector control strain. These data provide strong evidence that BspA and BspC, and thus possibly all Bsp family proteins, may facilitate vaginal colonisation by GBS through direct interactions with the vaginal mucosa. It was beyond the scope of this

project to identify the specific VEC receptor(s) involved. Nonetheless, studies with other streptococci do highlight a few potential candidates. As already mentioned, alike to other AgI/II family proteins, Bsp proteins may target ECM components. Another potential candidate is gp-340. Oral streptococci bind salivary glycoproteins to enable colonisation of soft and hard tissues of the oral cavity. AgI/II family proteins, specifically their A- and V-domains, mediate this attachment (Brady et al., 1992;Nakai et al., 1993) and specifically target gp-340, an innate immunity scavenger receptor protein. This has been shown for SpaP of *S. mutans*, SspA and SspB of *S. gordonii*, and AspA of *S. pyogenes*, as well as the Bsp proteins of GBS (Rego et al., 2016b;Leito et al., 2008;Maddocks et al., 2011). Gp-340 serves to aggregate bacteria for clearance when in fluid phase, yet can act as a receptor for adhesion when fixed to a host surface (Brady et al., 2010). Of note, gp-340 is cell-bound and highly expressed in the vaginal tract, and has been described to contribute to VEC interactions with another vaginal pathogen, HIV (Stoddard et al., 2007). As such, gp-340 may act as a colonisation determinant in the vaginal tract that is targeted by the Bsp proteins of GBS to promote colonisation. Bsp-mediated binding to gp-340 may also facilitate GBS colonisation of the oropharynx. This is particularly relevant to neonatal disease, for which colonisation of the throat is considered the route via which neonatal meningitis is initiated (Cheng et al., 2005b). In this regard, since the Bsp proteins have been shown to promote GBS internalisation into VECs, it would be of interest to determine if similar effects were seen with cells of the oropharynx. Similar to AspA, SpaP and SspB (Maddocks et al., 2011;Larson et al., 2010;Purushotham and Deivanayagam, 2014), the V domain of BspA has been implicated in binding gp-340 (Rego et al., 2016b). Thus, if the Bsp/gp-340 interaction was found to play a critical role in GBS colonisation and pathogenesis, it may be possible to elucidate the precise molecular basis of this interaction and so develop reagents that could impede this mechanism.

6.2.1.2 Interactions with *C. albicans* Als3

Following on from previous work showing that SspB of *S. gordonii* specifically associates with *C. albicans* Als3 (Silverman et al., 2010), a direct interaction between Als3 and GBS Bsp proteins was confirmed here. Moreover, the interaction between *C. albicans* adhesin Als3 and the GBS adhesins BspA or BspC appeared to play a critical role in the synergistic interaction between these two species. GBS showed a significant reduction in

coaggregation with *C. albicans*Δ*als3* when compared to the WT or complemented strains, and this was extended to the *L. lactis* BspA and BspC expressing strains, the interactions of which were virtually ablated with the *C. albicans*Δ*als3* strain. Even more strikingly, however, GBS was not promoted in VEC association when incubated with *C. albicans*Δ*als3* and likewise, *C. albicans* lacking Als3 was not enhanced in the presence of GBS. This suggests that Als3 is the sole target for GBS interactions with *C. albicans*, as has been implied for *S. gordonii* (Silverman et al., 2010), although (Klotz et al., 2007) showed that *S. gordonii* may also bind Als5. By contrast, enhanced VEC association was still seen for *C. albicans* when incubated with the 515Δ*bspC* strain, although this was by significantly lower than with the WT or complemented 515 strains. As such, it seems that although Als3 is the sole *C. albicans* target for GBS, Bsp proteins are not the sole GBS target for *C. albicans*.

Given the similarity of these data to previous work with *S. gordonii*, there may be a common mechanism for *C. albicans* Als3 interactions with streptococci. SspB has been inferred to bind the N-terminal peptide binding cavity of Als3, which has previously been shown to bind ECM proteins via their C-terminus, although the binding portion of SspB is as of yet unidentified (Hoyer et al., 2014;Lin et al., 2014;Bamford et al., 2015). In particular, *S. cerevisiae* cells expressing Als3 proteins deficient in N-terminal residues 166-225, 218-285, 270-305 and 277-286 were unable to form interactions with *S. gordonii* SspB (Bamford et al., 2015). It has been suggested that some SspB proteins undergo proteolytic cleavage to release the C-terminus from the peptidoglycan and that this then engages the Als3 peptide binding cavity (Hoyer et al., 2014). However, there are some significant differences in the C-termini of Bsp proteins compared to those AgI/II proteins of the oral streptococci. For example, in *S. mutans* SpaP, the C1-C2 portion of the protein is thought to have a role in binding to carbohydrates (Larson et al., 2011). However, Bsp proteins lack the C1 subdomain (Rego et al., 2016b). Furthermore, the C3 subdomain of Bsp proteins does not contain some of the key amino acids involved in metal ion binding that the C3 subdomains of other AgI/II proteins possess (Rego et al., 2016b), with the bound metal ions thought to confer increased stability and adhesion to ligands (Hall et al., 2014;Nylander et al., 2011;Forsgren et al., 2010). It is unclear then if such differences preclude a common binding mechanism. Future work could include domain mutagenesis studies to establish whether Bsp proteins and Als3 interact by a similar mechanism to SspB-Als3, including identifying if,

as with SspB, the N-terminal peptide binding domain of Als3 is the target for Bsp proteins.

6.2.1.3 Structure-function considerations

The Bsp proteins of GBS seemingly share a number of functional properties with the AgI/II family proteins of other streptococci, such as binding to gp-340 and *C. albicans*. Nonetheless, there are differences at the structural level that, in turn, could lead to functional variation (Figure 6-1). Differences in the C-termini of the Bsp and other AgI/II proteins have already been described. There are also critical differences relating to the V domain, in which the majority of inter- and intraspecies variation among other AgI/II family proteins lies (Brady et al., 2010). The V-domain of Bsp proteins has two anti-parallel β-sheets linked by loops and β-hairpins. This forms a β-sandwich core containing a large pocket consisting of negatively charged and hydrophobic residues, which is thought to be the region responsible for binding carbohydrates and glycoproteins (Rego et al., 2016b). In contrast, *S. mutans* SpaP V-domain is made up of 16 β-strands formed into two sheets, each consisting of eight antiparallel β-strands, and forms a lectin-like fold that presents a preformed ligand binding site (Troffer-Charlier et al., 2002). *S. gordonii* SspB possesses a similar V-domain fold, although the binding pocket is considerably smaller (Forsgren et al., 2009). Given these structural variations, it seems quite feasible that, while sharing some ligands, the Bsp proteins may also engage with receptors that differ from those utilised by the oral streptococci. To this end, it is worth noting that the V-domains of BspA and AspA, rather than exhibiting variation, share 70% sequence identity (Figure 6-1). Going forward, it will be of great interest to investigate if the V-domains of these proteins allow GBS and GAS to interact with ligands that directly relate to their classification as two of the 'true' pathogenic *Streptococcus* species. This could be achieved, for example, by utilising pull-down assays with the Bsp/AspA proteins and lysates or 'surfome' preparations of specific host cell types.

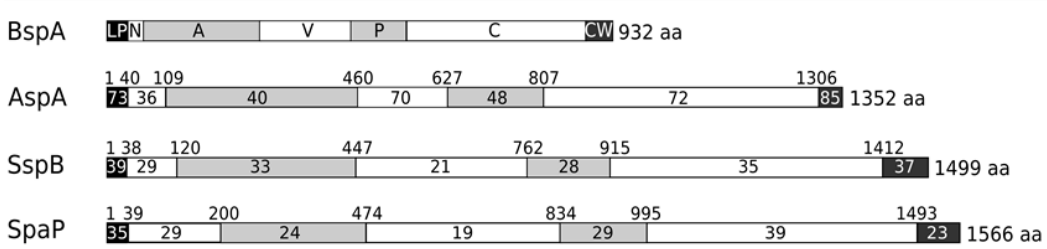

Figure 6-1 Domain similarity of AgI/II family proteins.

GBS BspA; GAS AspA; *S. gordonii* SspB; *S. mutans* SpaP. LP, leader peptide; N, N-terminal domain; A, alanine-rich repeats; V, variable-domain; P, proline-rich repeats; C, C-terminal domain; CW, cell wall anchorage region. Amino acid residue numbers are included, alongside the percentage identities of each domain relative to BspA. Adapted from (Rego, 2016).

Among the oral streptococci, it is typical for the A- and P-domains of AgI/II family proteins to coil around one another to form a 'stalk', with the V-domain presented at the 'tip' of the protein so that the ligand binding domain may protrude away from the surface of the bacterial cell (Larson et al., 2010). The Bsp A-domain lacks a heptad motif which is critical for this conformation in SpaP, SspB and AspA (Brady et al., 2010;Rego et al., 2016b). Nonetheless, it was found that the Bsp A- and P-domains still form a coiled-coil structure via an alternative mechanism, in which asparagine residues on the A-domain form hydrogen bonds with oxygen and nitrogen in the P-domain (Rego et al., 2016b). This conservation of structure implies its importance to adhesin functionality, and it has been proposed that the evolution of a stalk-like structure with an adhesive tip provides a level of flexibility that allows binding to be maintained under shear forces. As within the oral cavity, this would be relevant to microbes colonising the GU tract, further supporting a role for the Bsp proteins as colonisation determinants at this site. However, Bsp proteins are predicted to be significantly shorter than homologues of the oral streptococci i.e. 25-35 nm in length compared to 50 nm for SpaP (Rego et al., 2016b;Brady et al., 2010;Larson et al., 2010). This may suggest that Bsp proteins do not act to facilitate long-distance preliminary contact with host surfaces and indeed, this may not be necessary, as this role is likely undertaken by longer adhesins such as pili. This could then enable shorter adhesins such as Bsp to strengthen interactions subsequent to this.

6.2.1.4 Bsp variants and expression levels

There was some indication that different Bsp variants could modulate GBS interactions with VECs and *C. albicans*. NEM316, which carries both *bspA* and *bspB* genes bound

VECs at a higher level than 515, expressing just BspC. Likewise, *L. lactis* expressing BspA or BspC exhibited different VEC association levels. Of note, GBS strains 2603V/R and 18RS21, which did not coaggregate strongly with *C. albicans*, carry *bspD*. The sequence of BspD is identical to BspC, with the exception that it lacks the leader peptide that targets the protein to the translocation machinery. As such, these strains may fail to express a surface-bound form of BspD. It would be interesting to confirm this in future studies and investigate if lack of BspD expression affects the interactions of these strains with *C. albicans*. On a more general level, it will also be important to investigate more widely the Bsp expression profile of GBS strains and if this shows any correlation with disease profile. This could help inform, for example, if Bsp proteins may represent a potential therapeutic target to combat GBS disease. Chuzeville et al. (2015) found that of 75 GBS strains which possessed an AgI/II gene similar to *bspC*, only 40 contained signals for transcription or translation, suggesting that many of these proteins may not be expressed (Chuzeville et al., 2015). A further 42 GBS strains, additional to NEM316, were found to possess genes similar to *bspA* or *bspB*. The distribution of AgI/II genes among GBS strains differed depending on serotype and CC, with most strains associated with neonatal or adult disease possessing an AgI/II gene, while colonising strains did not (Chuzeville et al., 2015). Work presented here also inferred that expression of Bsp proteins may be growth-phase or temperature-dependent. Again, a more comprehensive analysis of this aspect using GBS reporter strains would be beneficial, as it could help identify the circumstances under which Bsp proteins play a critical role. This is particularly pertinent given the adhesin redundancy seen in GBS.

6.3 Effects of *C. albicans*-GBS interactions on host immune responses

Neutrophils are the predominant immune cell in the vaginal tract and levels of infiltration can significantly influence both microbial clearance and host tissue inflammation. It is of interest, therefore, that in these studies conditioned media from VEC coinfection appeared to be less chemotactic than from GBS monospecies infection. This is particularly striking, when considering that the microbial burden of coinfections was approximately 5- and 4-fold higher for GBS and *C. albicans* respectively. No strong chemotactic effects were seen for conditioned media from *C. albicans* monospecies infections. Thus, these data may indicate that upon coinfection, the *C. albicans* response

predominates, dampening the neutrophil response to GBS. Of note, similar effects were largely seen for induction of VEC cytokine gene transcription, with the major exception being IL-8 transcripts, the levels of which were significantly elevated upon coinfection. It must be acknowledged, however, that discrepancies exist within these data. IL-8 is a major neutrophil chemoattractant. Furthermore, *C. albicans* proteins SAP2 and SAP6 have been shown to trigger neutrophil chemotaxis by induction of IL-8 and MIP-2 from VECs (Gabrielli et al., 2016), and candidalysin has been shown to stimulate neutrophil migration to sites of infection (Richardson et al., 2017). Transcription of cytokine genes may not directly relate to subsequent protein expression levels. Thus exploring levels of cytokines released from VECs will be an important next step for these studies. It cannot be ruled out, however, that *C. albicans* effects on VECs are modulated by the presence of GBS and that this, in turn, leads to the differences from previous studies using *C. albicans* alone. Clearly then, much more research needs to be undertaken in this area but if coinfection does have a dampening effect on neutrophil recruitment, this could have a significant impact on *C. albicans* and GBS colonisation and pathogenesis within the GU tract. Neutrophil infiltration in response to *C. albicans* has been described as detrimental to the host, magnifying infection rather than providing a defensive role (Fidel et al., 2004), and subsequent inflammation within the GU tract does not correlate with neutralisation of *C. albicans* (Yano et al., 2012;Yano et al., 2014). Likewise, it has been suggested that GBS stimulation of neutrophils enhances inflammation and host damage rather than serving a protective role (Doran et al., 2002;Hensler et al., 2005). Reduced neutrophil recruitment upon coinfection might therefore minimise host tissue damage and inflammation, thereby promoting carriage within the GU tract.

Related to this were the effects of coinfection on major VEC signalling pathways involving MAPKs, as detected from the proteomics studies. These signalling pathways are important in terms of pathogenesis as they are involved in the initial recognition of microbes and signal to promote microbial clearance through such mediators as proinflammatory cytokines. MAPKs are activated specifically in response to *C. albicans* hyphae rather than blastospores (Moyes et al., 2010;Moyes et al., 2011), while GBS activates MAPKs following recognition by TLRs, and the resulting signalling cascade generates proinflammatory cytokines and induces ROS from resident and attracted immune cells (Henneke et al., 2002). Proteins relating to the MAPK pathways were, in general, significantly downregulated upon coinfection of VECs. For the *C. albicans* monospecies response, two MAPK-related proteins were upregulated and one

downregulated, while proteins only modulated upon coinfection showed downregulation of seven proteins involved in the MAPK pathway (and upregulation of none). This is particularly important given the promotion of microbial burden in contact with VECs for dual-species infections, which might be expected to enhance MAPK signalling. Again, these data imply that coinfection of VECs may dampen their proinflammatory response to the microbial challenge. It would be interesting to investigate whether this is a common mechanism, by repeating the proteomics studies with GBS strains 515 and COH1.

Taken together, these data suggest that *C. albicans* and GBS may operate synergistically to dampen the host proinflammatory response and so enhance their colonisation and persistence within the GU tract.

6.4 Therapeutic considerations

This work implicates Als3 and Bsp proteins as adhesins that likely contribute to coinfection of the vaginal epithelium by *C. albicans* and GBS. Going forward, identification of the precise interactions at the molecular scale of GBS or *C. albicans* adhesins with each other or with host receptors that contribute to GU tract colonisation could lead to development of novel anti-adhesive strategies to combat disease risk. There are a number of benefits to therapeutics that function by blocking adhesive interactions rather than by microbicidal mechanisms, as seen with more traditional drugs. Firstly, this approach would not exert a selective pressure that could contribute to the development of antimicrobial resistance. Moreover, anti-adhesive therapeutics are less likely to have side effects, and the resultant release of microbes from host surfaces would enhance the chances of microbial killing by immune cells (Ofek et al., 2003;Cozens and Read, 2012;Ternent et al., 2015). Such proof-of-concept has already been demonstrated for the AgI/II protein SpaP of *S. mutans*. A synthetic peptide that blocked the adhesive functions of SpaP successfully inhibited oral cavity colonisation by *S. mutans* (Kelly and Younson, 2000).

There is currently no effective GBS vaccine, and there have been numerous issues in developing a vaccine which would immunogenically cover the most common capsular serotypes associated with disease. This work implies that a vaccine targeting *C. albicans*

may also significantly reduce the burden and pathogenicity of GBS. There is a promising vaccine directed against recombinant Als3 which has passed phase 1 trials (Schmidt et al., 2012). This was developed to combat the synergistic partnership between *C. albicans* and *S. aureus*, which leads to an increase in *C. albicans* pathogenicity and devastating systemic *S. aureus* disease, often in immunocompromised individuals (Schlecht et al., 2015b). Studies with this vaccine have shown that vaccinated mice challenged with *C. albicans* orally, vaginally or intravenously were protected from disease. Additionally, mice were protected from systemic *S. aureus* infection (Schmidt et al., 2012). Phase 1 human clinical trials showed high levels of seroconversion, with production of IgG and IgA1 antibodies against Als3, as well as inducing production of IFN-γ and IL-17 (Schmidt et al., 2012). When this vaccine was investigated in a mouse model of VVC, anti-Als3 IgG serum and IgA vaginal antibodies were induced, resulting in a vast reduction in vaginal fungal burden (Ibrahim et al., 2013). As demonstrated for *S. aureus*, convergent immunity conferred by this Als3-based vaccine may therefore have the additional effect of reducing GU tract colonisation by GBS and thus risk of vertical transmission to initiate neonatal disease.

6.5 Additional future work

6.5.1 Alternative models of vaginal colonisation

The work detailed here describes, for the first time, a synergistic interaction between *C. albicans* and GBS that has potential to modulate interactions with VECs. Nonetheless, only early stage interactions could be explored, as extending the incubation period was associated with an increase in cytotoxicity and concomitant reduction in the integrity of the VEC monolayer. Furthermore, although the VK2/E6E7 cell line is widely used to examine vaginal tract interactions of microorganisms, and is largely comparable to native tissue, the original paper on VK2/E6E7 cell line generation notes that there is a slight difference in cytokeratin expression among these cells (Fichorova et al., 1997). Some of our microscopy studies using the nuclei stain DAPI also showed that certain 'giant' VECs possessed more than one nucleus (data not shown), which may have been due to the HPV E6 and E7 proteins promoting replication without successful cytokinesis. This VK2/E6E7 model is therefore appropriate for proof-of-principle studies, but more clinically-relevant options should be explored to take understanding of the GBS/*C.*

albicans interactions with vaginal epithelium further. One option would be to expand these studies using an *in vitro* reconstituted vaginal epithelial model such as that offered by Skin Ethics Laboratories. This incorporates cell differentiation and stratification and thus is more comparable to *in vivo* vaginal epithelium. Again, however, this would have limitations relating to time frame due to cytotoxicity, and this model also lacks other important host factors such as resident immune cells. Ultimately, it would be beneficial to validate the current findings using an *in vivo* model of vaginal colonisation. Murine models have been established for GBS (Patras et al., 2013) or *C. albicans* (Rahman et al., 2012) alone, which could likely be adapted for a coinfection scenario. The murine vaginal tract is similar to humans in that it is either persistently or sporadically colonised with GBS (Yow et al., 1980;Patras et al., 2013), but does differ with regards to a number of parameters, including pH, resident microbiota, oestrus cycle length and host response (Patras et al., 2013). Nevertheless, *in vivo* studies would provide important information about the contribution of GBS and *C. albicans* coinfection to colonisation over a longer time frame, and to the interplay of these microbes with the host immune response. Such models would also allow the possibility of coinfection modifying the invasive potential of these microbes and thus dissemination and systemic disease risk to be explored in greater detail.

6.5.2 Effects of other resident vaginal microorganisms

Lactobacilli are the dominant colonising commensal bacteria in the vaginal tract (Boris and Barbes, 2000). An inverse relationship has been reported between the presence of lactobacilli and GBS in the vaginal tract, suggesting that GBS colonisation requires dysbiosis (Ronnqvist et al., 2006;Kubota et al., 2002). Lactobacilli have been shown to outcompete GBS, preventing GBS proliferation and adhesion to host surfaces (Ruiz et al., 2012). In addition, vaginal challenge of mice with lactobacilli prior to inoculation with GBS protects the host from GBS-mediated infection by altering the immune response generated (De Gregorio et al., 2016). For *C. albicans*, the relationship with lactobacilli is less clear. Ronnqvist et al. (2006) and Demirezen (2002) found that vaginal burden of yeasts correlated with that of lactobacilli, although neither of these studies identified the yeast species, and lactobacilli were detected in over 89% of women identified as having VVC (Osset et al., 2001;Ronnqvist et al., 2006;Demirezen, 2002). By contrast, studies investigating lactobacilli as potential probiotics found that *Lactobacillus jensenii*

and *Lactobacillus johnsonii* showed high inhibition of *C. albicans* in broth culture (Gil et al., 2010), and that 8 out of 15 *Lactobacillus* strains tested significantly inhibited *C. albicans* association with VECs and provided a fungistatic effect in liquid culture (Osset et al., 2001). A further study found that *Lactobacillus reuteri* alone or in combination with *Lactobacillus rhamnosus* was able to significantly reduce the burden of *C. albicans* associating with VECs and induced greater levels of proinflammatory cytokines (Martinez et al., 2009). Such variations may reflect species- or strain-dependent differences.

Since at least certain lactobacilli seemingly have the capacity to modulate interactions of GBS or *C. albicans* with VECs, it would be of interest to investigate how GBS/*C. albicans* coinfection affects this dynamic. For example, if coinfection can cause dysbiosis due to inhibition of lactobacilli, or if the presence of lactobacilli can counteract the enhanced VEC association seen upon coinfection. This could be examined by adding lactobacilli to the VEC association assay, would help to gain further insight into GBS-*C. albicans* interactions in the natural GU tract environment.

6.5.3 Expansion of host response studies

Due to the limitations of the VEC model, this work focused on proinflammatory cytokines that are produced by epithelial cells. However, another important consideration with regards to the host response is those cytokines that are largely released by immune cells as part of the initial innate immune response. As mentioned, such data could be obtained from an *in vivo* coinfection model. As there were GBS strain-specific differences in terms of which cytokine genes were expressed, use of a wider panel of strains could also help to determine what the common GBS-*C. albicans* response is, and whether there are any correlations with GBS capsular serotype or CC. Transwell studies could be utilised to further explore whether infiltration of neutrophils is induced by *C. albicans*-GBS, and if this serves as a precursor to clearance of infection.

6.5.4 Intermicrobial responses

It is well documented that interactions between microorganisms can alter their respective proteomes. For example, in a multispecies biofilm with *S. gordonii* and *F. nucleatum*, *P. gingivalis* significantly decreased expression of proteins involved in cell

envelope formation and cell shape, while proteins involved in protein synthesis were upregulated (Kuboniwa et al., 2009). A further study into the interaction of *S. gordonii* with *F. nucleatum* or *P. gingivalis* showed that *S. gordonii* reacted to the interactions in a species-specific manner, i.e. different proteins were up- or downregulated depending on the binding partner (Hendrickson et al., 2012). When investigating the transcriptomics of an interspecies *C. albicans*-*S. gordonii* biofilm, (Dutton et al., 2016) found that genes involved in hyphae formation were upregulated, and thus *C. albicans* pathogenesis was promoted by *S. gordonii*. *S. gordonii* was less affected by the co-incubation (Dutton et al., 2016). It would be interesting therefore to explore whether similar responses were also seen upon *C. albicans*-GBS interaction, as this could provide further insight into the pathogenic potential of these microbes upon coinfection.

Additionally, it would be beneficial to expand the proteomics data to investigate the effects of coassociation on the microbial protein profile. To achieve this, a baseline profile of expression could be generated in blank K-SFM medium, and then compared against that of the microbe in the presence of VECs as a monospecies or dual-species infection. This could provide insight into whether synergism between *C. albicans* and GBS involves only modulation of the host environment, or also involves modulation of microbial protein expression to enable coassociation.

6.6 Conclusions

These studies show a synergism between *C. albicans* and GBS, leading to promotion of both species in association with VECs, both at the level of attachment and internalisation. The molecular basis for the *C. albicans*-GBS interaction was found to be dependent on candidal adhesin Als3 and involved, at least in part, direct binding by GBS Bsp proteins. Furthermore, Bsp proteins were identified as putative colonisation determinants for GBS within the GU tract by mediating direct interactions with vaginal epithelial cells. Identification of the importance of Als3 and Bsp surface adhesins, but particularly Als3, in mediating *C. albicans*-GBS interactions could have implications for future therapeutic interventions. For example, there is an anti-Als3 vaccine currently in clinical trials which could also serve to prevent GBS disease as a result of convergent immunity. There is data to suggest that GBS/*C. albicans* coinfection may dampen neutrophil recruitment, whilst modulating VEC proteins in a manner that could further

suppress the host proinflammatory response and promote microbial adhesion and internalisation. Taken together, such effects could promote colonisation of the vaginal mucosa by both *C. albicans* and GBS, and this, in turn, could enhance risk of disease by either opportunistic pathogen.

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Appendices

Appendix A: Proteomics

VEC proteins altered in expression in the GBS-only sample

Appendix 1 Overview of proteins that were upregulated only in the GBS-infected VECs relative to the VEC-only control.

Protein code	Protein name	Protein function	Relative abundance (VECs + GBS) / (VECs)
Q86V85	Integral membrane protein GPR180	G protein-coupled receptor	100
Q96P63	Serpin B12	Enzyme binding	2.871
P19013	Keratin, type II cytoskeletal 4	Keratin, expressed by epithelia, paired with keratin 13	2.67
A1A4E9	Keratin 13	Keratin, expressed by epithelia, paired with keratin 4	2.489
B4DL32	Keratin, type II cytoskeletal 5	Cytoskeletal organisation of actin, inhibits actin polymerisation	2.475
Q4KMP7	TBC1 domain family member	GTPase activating protein	2.298
O95455	dTDP-D-glucose 4,6-dehydratase	Metabolism	2.281
O60711	Leupaxin	Regulates cell adhesion	2.204
A0A087WUM0	SYNJ2BP-COX16 readthrough	Read-through transcription	2.186
Q08554	Desmocollin-1	Desmosome junction component, cell-cell adhesion	2.132
Q9BQF6	Sentrin-specific protease 7	De-sumolation	2.064

Appendix 2 Overview of proteins that were downregulated only in the GBS-infected VECs relative to the VEC-only control.

Protein code	Protein name	Protein function	Relative abundance (VECs + GBS) / (VECs)
Q13535	Serine/threonine-protein kinase ATR	DNA damage sensor, serine/threonine protein kinase	0.297

Protein code	Protein name	Protein function	Relative abundance (VECs + GBS) / (VECs)
Q8WWQ0	PH-interacting protein	Cytoskeletal organisation	0.37
Q53EY2	ElaC homolog 1 variant	RNA transport	0.377
B3KQT6	Tetraspanin-13	Signal transduction, membrane protein	0.405
M1VKI3	Tyrosine-protein kinase receptor	Protein tyrosine kinase receptor, membrane protein	0.42
P78527	DNA-dependent protein kinase catalytic subunit	Serine/threonine protein kinase	0.427
O60318	Germinal-center associated nuclear protein	DNA replication	0.427
A8K2P6	Zinc finger protein 503	Possible transcriptional repressor	0.43
A0A0S2Z2Z6	Annexin	Vesicle fusion	0.445
P51636	Caveolin-2	Scaffolding protein	0.45
O75643	U5 small nuclear ribonucleoprotein 200 kDa helicase	RNA helicase	0.452
P52701	DNA mismatch repair protein Msh6	Mismatch repair	0.453
Q9Y2X0	Mediator of RNA polymerase II transcription subunit 16	Transcriptional regulation	0.461
A0A0A0MTH9	TATA-binding protein-associated factor 172	Transcriptional regulation	0.468
Q9HB20	Pleckstrin homology domain-containing family A member 3	Intracellular trafficking	0.468
Q13137	Calcium-binding and coiled-coil domain-containing protein 2	Targetting bacteria to autophagosome, cytoskeletal organisation of actin	0.476
A8K359	Excision repair cross-complementing rodent repair deficiency	DNA helicase	0.496
E9PLM6	Midkine	Growth factor	0.499
B7ZKS3	Ubiquitin specific peptidase 48	Deubiquitinating enzyme	0.499

VEC proteins altered in expression in the *C. albicans*-only sample

Appendix 3 Overview of proteins that were upregulated only in the *C. albicans*-infected VECs relative to the VEC-only control.

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)
A0A0A0MQX1	Unconventional myosin-X	Myosin, actin binding	3.068
Q8N0Y7	Probable phosphoglycerate mutase 4	Enzyme, glycolytic process	2.59
I3L3D5	Profilin	Cytoskeletal organisation of actin	2.441
Q9HAT2	Sialate O-acetylesterase	Enzyme, metabolic process	2.434
Q9BZQ6	ER degradation-enhancing alpha-mannosidase-like protein 3	Endoplasmic reticulum protein degredation	2.401
P20749	B-cell lymphoma 3 protein	Activation of NF-kappa-B	2.378
P61225	Ras-related protein Rap-2b	Signal transduction, cytoskeletal organisation	2.339
A0A024R4Y2	HCG39762	Possible transmembrane transport	2.331
A0A0G2JMZ8	Occludin	Formation of tight junctions, membrane protein	2.247
A8K3H9	v-rel reticuloendotheliosis viral oncogene homolog B	NF-kappa-B subunit	2.221
A0A0S2Z3C0	Actinin alpha 4 isoform 3	Cross links actin, vesicle transport	2.211
P16519	Neuroendocrine convertase 2	Processing of hormones	2.165
A0A0M4UT83	Glutathione S-transferase P	Enzyme	2.161
Q9H488	GDP-fucose protein O-fucosyltransferase 1	Glycosyltransferase	2.15
P27701	CD82 antigen	Associates with CD4 or CD8 and delivers costimulatory signals for the TCR/CD3 pathway.	2.138
Q8IXL7	Methionine-R-sulfoxide reductase B3	Enzyme	2.127
P02452	Collagen alpha-1(I) chain	Collagen	2.116
Q4ZGM8	Hemoglobin alpha-2 globin mutant	Haemoglobin	2.113
A8K556	cDNA FLJ78217	Homeostasis of epithelial cells	2.069
F8WES2	S-methyl-5'-thioadenosine phosphorylase	Enzyme	2.069
D6RFX5	Amphiregulin	Promotes growth of epithelial cells	2.069

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)
Q96HE7	ERO1-like protein alpha	Enzyme	2.065
E7EX59	Propionyl-CoA carboxylase beta chain, mitochondrial	Enzyme	2.06
Q59EQ5	Cysteine and glycine-rich protein 1 variant	Metal ion binding	2.053
H0Y6T8	Ras-related protein Rab-18	GTPase	2.053
P23284	Peptidyl-prolyl cis-trans isomerase B	Enzyme, protein folding	2.047
A0A1W2PRS1	Lysosome membrane protein 2	Lysosome membrane protein	2.035
A8K9L8	Tropomodulin 2	Cytoskeletal organisation of actin	2.034

Appendix 4 Overview of proteins that were downregulated only in the *C. albicans*-infected VECs relative to the VEC-only control.

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)
Q86Z14	Beta-klotho	Transcriptional repressor	0.146
Q15329	Transcription factor E2F5	Transcription factor, cell proliferation	0.319
D6RH31	Nephronectin	Possible calcium binding protein	0.344
Q8TF42	Ubiquitin-associated and SH3 domain-containing protein B	Promotes localisation of activated EGFR to membrane	0.346
Q8TDG2	Actin-related protein T1	Spermatid formation	0.353
A0A024QZD9	Zinc finger protein 668	Nucleic acid binding	0.368
Q9P0M6	Core histone macro-H2A.2	Histone, transcriptional regulation	0.374
A0A024R9Z8	Aryl hydrocarbon receptor	Transcription factor	0.394
Q3KQV9	UDP-N-acetylhexosamine pyrophosphorylase-like protein 1	Metabolism	0.396
Q99808	Equilibrative nucleoside transporter 1	Nucleoside transporter, membrane protein	0.399
Q8IZX4	Transcription initiation factor TFIID subunit 1-like	Meiosis	0.405
Q07092	Collagen alpha-1(XVI) chain	Collagen	0.409

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)
Q96L73	Histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific	Histone methylation	0.417
Q15904	V-type proton ATPase subunit S1	ER membrane trafficking	0.417
Q8WY91	THAP domain-containing protein 4	Possible DNA binding protein	0.451
Q6P0N6	DST protein	Cytoskeletal linker protein	0.454
O75808	Calpain-15	Transcription factor	0.46
B2C310	Glutathione S-transferase pi	Enzyme, transferase	0.463
A0A024R5D2	Multiple endocrine neoplasia I	Transcriptional regulation	0.466
O94906	Pre-mRNA-processing factor 6	Processing of pre-mRNAs	0.472
Q14684	Ribosomal RNA processing protein 1 homolog B	Induces apoptosis in response to DNA damage	0.473
B2RB17	F-box protein 5	Regulates cell cycle	0.478
A0A024RDT9	Fibronectin type III domain containing 3A	Spermatogenesis	0.481
Q53GA4	Pleckstrin homology-like domain family A member 2	Uncharacterised	0.483
P54278	Mismatch repair endonuclease PMS2	DNA mismatch repair	0.484
A0A0S2Z570	RXRB	Retinoic acid receptor	0.484
B2RAF2	cDNA, FLJ94875	Uncharacterised	0.485
O75182	Paired amphipathic helix protein Sin3b	Transcriptional repressor	0.487
Q8TEC9	cDNA FLJ23639 fis	RNA helicase	0.49
A0A024R377	KIAA0863 protein	Uncharacterised	0.49
P08779	Keratin, type I cytoskeletal 16	Keratin	0.493
Q13835	Plakophilin-1	Cytoskeletal linker protein	0.493
Q549H9	cAMP-dependent protein kinase inhibitor	Inhibitor of cAMP-dependent protein kinases	0.494
Q8IZW8	Tensin-4	Possible promoter of apoptosis, signal transduction	0.496

VEC proteins altered in expression in the *C. albicans* and *C. albicans*+GBS samples

Appendix 5 Overview of proteins that were upregulated in the *C. albicans*-infected VECs and *C. albicans*+GBS-infected VECs relative to the VEC-only control.

Protein code	**Protein name**	**Protein function**	**Relative abundance (VECs + *C. albicans*) / (VECs)**	**Relative abundance (VECs + *C. albicans* + GBS) / (VECs)**
Q8TEY4	Adaptor protein FE65a2	Nuclear protein, transcriptional regulation	34.086	35.737
Q71F56	Mediator of RNA polymerase II transcription subunit 13-like	Mediates transcription	25.207	30.502
Q6MZX7	Uncharacterized protein DKFZp686M24218	Uncharacterised	12.857	11.247
X5D8U4	Arginine vasopressin isoform A	Hormone	9.83	12.45
Q1RMG2	Adenosylhomocysteinase	Methylation control	7.408	7.724
H0YL33	Annexin	Ion transport	6.407	3.609
P02790	Hemopexin	Iron regulation	6.214	6.391
Q7Z351	Uncharacterized protein DKFZp686N02209	Uncharacterised	5.871	5.937
B4DRF2	Complement factor I	Regulates complement cascade	5.619	5.113
P02787	Serotransferrin	Iron transport, membrane protein	5.582	5.758
B4E1B2	cDNA FLJ53691, highly similar to Serotransferrin	Iron transport	5.286	5.657
I3WAC9	Insulin	Hormone	5.275	5.449
Q9BX10	GTP-binding protein 2	GTP-binding protein	4.987	5.628
P10645	Chromogranin-A	Antimicrobial peptide	4.833	5.083
B2R892	cDNA, FLJ93793, highly similar to Homo sapiens creatine kinase, muscle	Enzyme	4.789	4.018
Q6ICB4	Sesquipedalian-2	Intracellular trafficking	4.681	2.705

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
U3PXP0	Alpha globin chain	Oxygen transport	4.357	4.591
P01100	Proto-oncogene c-Fos	Signal transduction, cell proliferation	4.354	4.501
A0A161I202	Lactoferrin	Antimicrobial peptide, iron binding	4.235	4.912
A0A087WXL3	DNA polymerase theta	DNA Polymerase	4.026	4.578
Q96IZ5	RNA-binding protein 41	RNA binding	3.891	4.611
A0A024R3E3	Apolipoprotein A-I	Apolipoprotein	3.859	4.191
Q9Y2K1	Zinc finger and BTB domain-containing protein 1	Transcriptional repressor	3.849	4.652
Q6ZN40	Tropomyosin 1 (Alpha)	Actin binding protein	3.76	4.754
Q14403	Gamma-G globin	Haemoglobin	3.652	3.443
B4DPP6	cDNA FLJ54371, highly similar to Serum albumin	Blood protein	3.52	3.35
A0A0F7RQP6	Thyroid stimulating hormone beta subunit	Hormone control	3.337	4.154
B2R7D2	cDNA, FLJ93389, highly similar to Homo sapiens multiple inositol polyphosphate histidine phosphatase 1	Enzyme	3.272	2.602
B2R5M3	cDNA, FLJ92530, highly similar to Homo sapiens chromogranin B (secretogranin 1)	Uncharacterised	3.214	3.696
A0A024R755	Calumenin	Protein folding	3.209	6.516
I3NI25	LisH domain-containing protein FOPNL	Cilia biogenesis	3.193	3.371
Q01650	Large neutral amino acids	Amino acid transport	3.134	3.233

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	transporter small subunit 1			
A0A0B4J1S4	Selenoprotein F	Protein folding	3.126	3.869
P53985	Monocarboxylate transporter 1	Membrane transporter, membrane protein	3.09	3.349
E5RJP0	Focal adhesion kinase 1	Cytoskeletal organisation, MAPK activation	3.01	3.727
J3QL06	Hypoxia up-regulated protein 1	Protein folding	2.963	4.463
P05026	Sodium/potassium-transporting ATPase subunit beta-1	ATPase, cell adhesion, membrane protein	2.935	3.061
A0A024R5W6	Tropomyosin 1 (Alpha)	Actin binding protein	2.931	3.413
Q5SQI5	Neuroepithelial cell transforming gene 1	Interacts with RhoA GTPase	2.868	2.743
U5XK60	Sex-determining region Y protein	Transcriptional regulation	2.833	2.634
B4DE27	cDNA FLJ58765, highly similar to Neutral amino acid transporter B(0)	Amino acid transport	2.825	3.781
P05023	Sodium/potassium-transporting ATPase subunit alpha-1	ATPase, ion transport	2.823	3.162
A1E282	Beta-actin	Actin	2.803	2.384
A0A024R8U1	Solute carrier family 16 (Monocarboxylic acid transporters), member 3	Membrane transporter, membrane protein	2.797	2.762
Q63HJ5	Uncharacterized protein DKFZp686L2367	Uncharacterised	2.786	2.413
O15061	Synemin	Muscle support	2.753	3.977
Q15293	Reticulocalbin-1	Calcium ion binding	2.742	3.358

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
A0A024R5M9	Nuclear mitotic apparatus protein 1	Mitotic spindle organisation	2.708	3.274
B2R6W9	cDNA, FLJ93157, highly similar to Homo sapiens SH3KBP1 binding protein 1	Uncharacterised	2.686	2.479
Q8N5A0	Eukaryotic translation initiation factor 5B	Translation initiator	2.683	5.259
P48509	CD151 antigen	Cell adhesion, integrin binding, membrane protein	2.682	2.812
V9HW63	Epididymis secretory sperm binding protein Li 97n	Regulates NF-kappa-B	2.681	3.219
A0A1K0GXZ1	Globin C1	Ion binding	2.649	2.43
A0A024R895	SET translocation (Myeloid leukemia-associated)	Apoptosis, transcription	2.642	3.015
E7EWM2	Centrosomal protein of 170 kDa	Cytoskeletal organisation	2.634	3.096
A0A087WVV1	Proteasome subunit beta type-2	Component of the proteosome	2.623	2.685
A0A024R5R0	KIAA0256 gene product	Uncharacterised	2.621	4.323
Q15758	Neutral amino acid transporter B(0)	Amino acid transport	2.617	2.768
Q8IWA5	Choline transporter-like protein 2	Uncharacterised	2.614	2.781
Q96AE7	Tetratricopeptide repeat protein 17	Actin polymerisation for ciliation	2.605	2.188
Q4KWH8	1-phosphatidylinositol 4,5-bisphosphate	Enzyme	2.598	2.465

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	phosphodiesterase eta-1			
P18084	Integrin beta-5	Cell adhesion, membrane protein	2.585	2.484
P16870	Carboxypeptidase E	Processes hormones	2.577	2.643
P08123	Collagen alpha-2(I) chain	Collagen	2.553	2.296
Q6N030	Uncharacterized protein	Uncharacterised	2.552	2.747
U3KQV3	Unnamed protein	GTPase, membrane protein	2.536	3.125
P51884	Lumican	Collagen binding and organisation	2.522	2.704
G9FP35	Guanine nucleotide binding protein	Nucleotide binding	2.507	2.561
P62633	Cellular nucleic acid-binding protein	Nucleic acid binding, transcriptional regulation	2.489	3.791
Q6LAF9	Cathepsin B	Protein degradation	2.471	2.932
A0A087WWZ6	PR domain-containing protein 11	Transcriptional regulation	2.464	2.328
A0A024R397	Pannexin	Component of gap junctions, membrane protein	2.456	2.425
A0A140VK08	Testicular secretory protein Li 8	Calcium ion binding	2.43	2.845
B7ZKJ8	ITIH4 protein	Response to surgical trauma	2.427	2.107
A0A5E4	Uncharacterized protein	Uncharacterised	2.425	2.692
V9HWA9	Epididymis secretory sperm binding protein Li 62p	Complement C3, antimicrobial activity	2.409	2.14
Q5NDL2	EGF domain-specific O-linked N-acetylglucosamine transferase	Enzyme	2.392	2.18
G8JLH6	Tetraspanin	Cell surface protein, membrane protein	2.39	2.063

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
U3KQE2	Calpain small subunit 1	Cytoskeletal organisation, influences apoptosis proteins, adhesion	2.385	3.808
Q5U0D2	Transgelin	Actin binding protein	2.377	2.221
P62847	40S ribosomal protein S24	Ribosomal component	2.367	5.66
A8K0V5	cDNA FLJ77004, highly similar to Homo sapiens N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 4	Enzyme	2.363	2.379
A0A024R3R5	Lamin B receptor	Nuclear membrane integrity	2.352	5.03
Q6IAW5	CALU protein	Protein folding	2.35	2.845
A0A024R884	Calponin	ECM protein	2.339	2.827
Q53FP8	Tenascin C (Hexabrachion)	Smooth muscle contraction, actin binding	2.339	3.465
D6RAN1	PDZ and LIM domain protein 7	Actin binding protein	2.336	2.26
Q13438	Protein OS-9	Protein degredation	2.329	3.115
B4DWN9	cDNA FLJ55258, highly similar to Homo sapiens pleckstrin homology domain containing, family F member 1	Limited information	2.326	2.622
F5GZS6	4F2 cell-surface antigen heavy chain	Ion transport, membrane protein	2.323	2.375
P53801	Pituitary tumor-transforming gene 1 protein-interacting protein		2.323	2.009
Q14257	Reticulocalbin-2	Calcium ion binding	2.295	3.119
Q5JTV8	Torsin-1A-interacting protein 1	Nuclear membrane integrity	2.29	2.623

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q9Y4L1	Hypoxia up-regulated protein 1	Protein folding	2.286	2.603
P54709	Sodium/potassium-transporting ATPase subunit beta-3	ATPase, membrane protein	2.275	2.304
Q59G10	Aldehyde dehydrogenase 1 family, member L1 variant	Regulates apoptosis	2.266	2.472
Q14473	Uncharacterized protein	Uncharacterised	2.264	2.579
P19256	Lymphocyte function-associated antigen 3	T cell activation, membrane protein	2.262	2.548
P07204	Multiple coagulation factor deficiency protein 2	Endothelial receptor	2.247	2.14
Q8NI22	Thrombomodulin	ER-Golgi transport	2.247	2.832
B4DW33	cDNA FLJ54187, highly similar to Homo sapiens polymerase (RNA) I associated factor 1	RNA Polymerase	2.235	2.297
Q14894	Ketimine reductase mu-crystallin	Catalytic enzyme	2.232	2.093
H7BY55	Complement decay-accelerating factor	Inhibits complement cascade, membrane protein	2.23	2.403
Q9UGJ0	5'-AMP-activated protein kinase subunit gamma-2	ATP binding	2.221	2.862
B2RAH2	Sodium/hydrogen exchanger	Membrane transporter, expressed in kidney and intestine	2.221	2.302
Q8IUW5	RELT-like protein 1	Uncharacterised, membrane protein	2.221	2.11

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
P16070	CD44 antigen	Mediates cell-cell and cell-matrix interactions, membrane protein	2.208	2.394
P13521	Secretogranin-2	Chemotactic protein	2.208	2.337
Q9H3Z4	DnaJ homolog subfamily C member 5	Chaperone	2.2	2.386
H7C0W7	Hyccin	Possible role in the beta-catenin/Lef signaling pathway	2.197	2.21
A0A024R2Z1	Guanine nucleotide binding protein (G protein)	Light perception	2.187	2.844
Q9H7U1	Serine-rich coiled-coil domain-containing protein 2	Microtubule binding	2.186	2.881
Q8WWX9	Selenoprotein M	Metabolism	2.186	2.084
A0A1S5UZH5	Mitochondrial thioredoxin	Redox reactions	2.185	2.119
E7ESP4	Integrin alpha-2	Cell adhesion, ECM protein receptor, membrane protein	2.177	2.563
Q8NC44	Reticulophagy regulator 2	Uncharacterised, membrane protein	2.17	2.791
Q99549	M-phase phosphoprotein 8	Histone binding	2.163	2.444
V9HWB4	Epididymis secretory sperm binding protein Li 89n	Protein folding	2.162	2.658
A0A0F7G8J1	Plasminogen	Degrades blood clots	2.16	2.133
Q8NI35	InaD-like protein	May regulate protein targeting, cell polarity and integrity of tight junctions, membrane protein	2.143	2.321
Q9Y4G2	Pleckstrin homology domain-	Regulation of endosomal trafficking	2.131	3.123

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	containing family M member 1			
H7BZJ3	Protein disulfide-isomerase A3	Protein folding	2.129	2.513
P00352	Retinal dehydrogenase 1	Enzyme	2.128	2.13
E9PN89	Heat shock cognate 71 kDa protein	Protein folding	2.12	3.91
Q9UBS4	DnaJ homolog subfamily B member 11	Protein folding	2.116	2.584
P05556	Integrin beta-1	Cell adhesion, membrane protein	2.106	2.384
Q03405	Urokinase plasminogen activator surface receptor	Plasmin formation, membrane protein	2.104	2.449
A0A024R206	Ring finger protein 126	Ubiquitination	2.099	3.212
Q9Y639	Neuroplastin	Cell adhesion, activation of p38 MAPK, membrane protein	2.094	2.38
B4DL55	Laminin beta-3 chain	Laminin	2.084	2.395
P62979	Ubiquitin-40S ribosomal protein S27a	Ubiquitin, ribosome component	2.068	2.016
A0A024RA75	3-hydroxyisobutyrate dehydrogenase	Enzyme	2.066	2.232
P05362	Intercellular adhesion molecule 1	Signalling receptor activity, integrin binding, membrane protein	2.063	2.217
Q59EN5	Prosaposin variant	Lysosomal protein	2.054	2.386
Q7Z406	Myosin-14	Cytokinesis	2.053	2.348
A0A1U9X8X6	CDSN	Epidermal protein	2.046	2.82
A0A024R2F8	Coiled-coil-helix-coiled-coil-helix domain containing 4	Chaperone	2.045	2.456

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
M0R344	Sphingosine kinase 2	Enzyme	2.043	2.143
H0YMT9	Annexin	Ion transport	2.031	2.391
K7EKQ2	Hsp90 co-chaperone Cdc37	Chaperone, interacts with kinases	2.027	2.004
V9HWD3	Epididymis luminal protein 117	Degradation of improperly folded proteins	2.025	2.275
B3KW93	Sodium/potassium-transporting ATPase subunit alpha	Ion transport	2.024	2.937
A0A024RD07	Trinucleotide repeat containing 5	Chaperone, protein folding of TLRs	2.021	2.249
Q8IXB1	DnaJ homolog subfamily C member 10	Chaperone, protein folding	2.016	2.877
Q14696	LDLR chaperone MESD	Chaperone	2.013	2.206
Q59FZ8	Nebulette non-muscle isoform variant	Metal ion binding	2.002	2.176

Appendix 6 Overview of proteins that were downregulated in the *C. albicans*-infected VECs and *C. albicans*+GBS-infected VECs relative to the VEC-only control.

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
E7EW20	Unconventional myosin-VI	Intracellular transport	0.01	0.01
A0A0A0MT16	ATP-binding cassette sub-family A member 13	ATP binding	0.01	0.01
A0A024QZE6	Chromosome 16 open reading frame 58	Membrane protein	0.01	0.01
A8K5Y2	cDNA FLJ77228, highly similar to Homo sapiens amphiphysin		0.057	0.082

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
C9JXG8	Ran-specific GTPase-activating protein	Intracellular transport	0.07	0.109
P01036	Cystatin-S	Protease inhibitor	0.079	0.161
A4D198	Similar to mKIAA0038 protein	RNA binding	0.087	0.143
Q9UHN6	Cell surface hyaluronidase	Enzyme	0.093	0.068
Q0P5N8	TMSB4X protein	Cytoskeletal organisation of actin, inhibits actin polymerisation	0.11	0.059
Q8TDS5	Oxoeicosanoid receptor 1	Fatty acid receptor	0.116	0.161
Q96FS1	CTNND1 protein	Cell adhesion, signal transduction	0.123	0.237
Q6UW63	KDEL motif-containing protein 1	Glucosyltransferase	0.125	0.23
B7Z9D5	cDNA FLJ50387	rRNA processing	0.141	0.134
J3KP58	CAP-Gly domain-containing linker protein 1	Cytoskeletal organisation of microtubules	0.147	0.155
Q9HD40	O-phosphoseryl-tRNA(Sec) selenium transferase	Enzyme	0.148	0.401
Q92736	Ryanodine receptor 2	Calcium channel, membrane protein	0.153	0.143
I3L1P8	Mitochondrial 2-oxoglutarate/malate carrier protein	Mitochondrial transport	0.155	0.09
M0R2C6	Uncharacterized protein	Uncharacterised	0.162	0.091
E9PLG2	26S proteasome regulatory subunit 6A	Proteosome	0.169	0.095
Q13232	Nucleoside diphosphate kinase 3	Nucleotide triphosphate synthesis	0.177	0.143
F5H7B7	ATP-binding cassette sub-	ATP binding	0.188	0.206

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	family A member 13			
X6R7I6	Pancreatic progenitor cell differentiation and proliferation factor	Uncharacterised	0.189	0.172
Q08AJ9	Histone H2A	Histone	0.193	0.366
Q9H6N6	Putative uncharacterized protein MYH16	Myosin	0.207	0.167
Q3BDU4	Rhabdomyosarcoma antigen MU-RMS-40.10B	DNA mismatch repair	0.208	0.201
A0A090N8E9	Enhancer of zeste homolog 2	Transcriptional regulation	0.211	0.217
E7EMW0	Centrosomal protein of 170 kDa	Cytoskeletal organisation of microtubules	0.214	0.149
F8VWW7	SPRY domain-containing protein 3	Uncharacterised	0.219	0.183
Q9HD33	39S ribosomal protein L47, mitochondrial	Ribosomal protein	0.239	0.156
A0A1U9X819	MRPS18B	Mitochondrial ribosome protein	0.256	0.145
B7Z7L8	cDNA FLJ50425, highly similar to Bardet-Biedl syndrome 4 protein	Pseudogene	0.258	0.348
B4DSG5	Tax1-binding protein 1	Inhibits apoptosis, inflammatory response	0.26	0.219
Q68CZ1	Protein fantom	Embryonic development, may be involved in apoptosis	0.26	0.393
A6NGR9	Maestro heat-like repeat-containing protein family member 6	Uncharacterised	0.264	0.155
A0A024RAP2	3-hydroxy-3-methylglutaryl	Cholestorol biosynthesis	0.267	0.375

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	coenzyme A reductase			
A8K883	cDNA FLJ77590, highly similar to Homo sapiens leucine rich repeat containing 1	Uncharacterised	0.269	0.268
B2R8G6	Peptidylprolyl isomerase	Protein folding	0.273	0.315
Q8N0Z6	Tetratricopeptide repeat protein 5	DNA binding	0.275	0.258
B7ZKN5	UTX protein	Regulation of gene expression	0.29	0.188
B2R636	cDNA, FLJ92762, highly similar to Homo sapiens ubiquitin specific protease 1	DNA repair	0.294	0.401
D3DQ70	SERPINE1 mRNA binding protein 1	mRNA stability	0.308	0.267
Q9P0C6	HSPC255	Activates MAPK	0.31	0.339
A8K8V8	cDNA FLJ76444	Chaperone	0.312	0.383
Q15438	Cytohesin-1	Membrane transport, membrane protein	0.313	0.276
Q96EB3	EEF1A1 protein	Translation	0.316	0.416
Q7Z6V5	tRNA-specific adenosine deaminase 2	tRNA modification	0.33	0.153
P57768	Sorting nexin-16	Intracellular transport	0.331	0.332
Q14680	Maternal embryonic leucine zipper kinase	Apoptosis, cell cycle regulation	0.331	0.247
Q14690	Protein RRP5 homolog	Involved in the biogenesis of rRNA.	0.335	0.245
Q14469	Transcription factor HES-1	Transcriptional regulation	0.342	0.24
M0QX27	Urokinase plasminogen activator surface receptor	Plasmin formation	0.345	0.202

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
O75676	Ribosomal protein S6 kinase alpha-4	Intracellular signalling, regulation of NF-kappa-B, inflammatory response	0.351	0.342
Q01546	Keratin, type II cytoskeletal 2 oral	Intermediate filament, cytoskeletal protein	0.352	0.322
A8K800	cDNA FLJ76924, highly similar to Homo sapiens brix domain containing 1	Ribosome assembly	0.361	0.407
A1JUI8	Chaperonin subunit 6A	Chaperone	0.362	0.346
O75529	TAF5-like RNA polymerase II p300/CBP-associated factor-associated factor 65 kDa subunit 5L	Transcriptional regulation	0.365	0.433
Q6S5L8	SHC-transforming protein 4	Tyrosine kinase binding, may have a role in apoptosis, membrane protein	0.365	0.323
F8VW41	Phosphofurin acidic cluster sorting protein 2	Apoptosis, ion trafficking	0.366	0.35
A0A024R509	Arylsulfatase A	Enzyme	0.367	0.333
B2R5U4	Suppressor of fused homolog	Transcriptional regulation	0.372	0.431
A0A024R1U1	PSMC3 interacting protein	Reciprocal meiotic recombination	0.374	0.386
A0A024RA53	Serine/threonine kinase 17a (Apoptosis-inducing)	Induces apoptosis	0.376	0.429
Q15004	PCNA-associated factor	DNA replication, regulates DNA repair	0.378	0.262
A0A1W2PP10	Vacuolar protein sorting-associated protein 35	Intracellular transport	0.379	0.352

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
B4DIS6	cDNA FLJ56443, highly similar to Putative ATP-dependent RNA helicase DHX33	ATP binding, helicase activity	0.38	0.379
A8K4P8	cDNA FLJ75337	Uncharacterised	0.381	0.48
Q9H6R4	Nucleolar protein 6	rRNA processing	0.382	0.448
A0A087WZ30	ATP-dependent DNA helicase Q4	DNA helicase	0.383	0.438
Q59EJ3	Heat shock 70kDa protein 1A variant	Protein folding	0.386	0.457
A0A126LAX6	U30	Virion assembly	0.388	0.3
Q9HC06	Cd002 protein	Intracellular transport	0.393	0.226
H0Y5K5	Endoplasmic reticulum-Golgi intermediate compartment protein 3	Intracellular transport	0.394	0.279
Q14593	Zinc finger protein 273	Transcriptional regulation	0.395	0.49
Q7L3V2	Protein Bop	Induces apoptosis	0.401	0.486
Q9UQR0	Sex comb on midleg-like protein 2	Transcriptional regulation	0.404	0.409
P02533	Keratin, type I cytoskeletal 14	Intermediate filament, cytoskeletal protein	0.406	0.388
A6NKB5	Pecanex-like protein 2	Possible role in tumorigenesis	0.406	0.298
Q9Y3B7	39S ribosomal protein L11, mitochondrial	Mitochondrial ribosome protein	0.408	0.439
H3BQK0	ATP-dependent RNA helicase DDX19B	RNA helicase	0.412	0.392
E7EXA6	Chromosome transmission fidelity protein 18 homolog	Chromosome cohesion	0.413	0.369
A8KAK5	cDNA FLJ77399, highly similar to Homo sapiens cofactor required for Sp1	Activates transcription	0.416	0.499

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
		transcriptional activation		
Q9H977	WD repeat-containing protein 54	Uncharacterised	0.416	0.312
O75063	Glycosaminoglycan xylosylkinase	Kinase activity	0.416	0.331
Q05CW7	NAT10 protein	Chromatin organisation	0.417	0.487
Q86YS6	Ras-related protein Rab-43	Intracellular transport, membrane protein	0.418	0.461
B9EGE7	ZNF507 protein	Transcriptional regulation	0.418	0.493
A0A0A0MST0	Myeloid differentiation primary response protein MyD88	MyD88, inflammatory response, upregulation of cytokines and chemokines, NF-kappa-B activation	0.419	0.365
P26045	Tyrosine-protein phosphatase non-receptor type 3	Protein tyrosine phosphatase, membrane protein	0.42	0.375
A8K2I0	Keratin 6A	Intermediate filament, cytoskeletal protein	0.424	0.379
O75319	RNA/RNP complex-1-interacting phosphatase	RNA binding	0.424	0.427
K7EMC2	Rho GTPase-activating protein 33	GTPase activator, intracellular trafficking	0.426	0.383
O15195	Villin-like protein	Actin binding	0.426	0.45
Q969X5	Endoplasmic reticulum-Golgi intermediate compartment protein 1	Intracellular transport	0.428	0.312
Q53RE8	Ankyrin repeat domain-containing protein 39	Uncharacterised	0.43	0.395
Q53HE6	HSPC163 protein variant	Intracellular transport	0.432	0.325

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q9NQ88	Fructose-2,6-bisphosphatase TIGAR	Enzyme	0.434	0.296
P35080	Profilin-2	Cytoskeletal organisation of actin, inhibits actin polymerisation	0.435	0.462
Q5VZL5	Zinc finger MYM-type protein 4	Cytoskeletal organisation	0.435	0.472
P47224	Guanine nucleotide exchange factor MSS4	Vesicular transport	0.437	0.481
P84157	Matrix-remodeling-associated protein 7	Uncharacterised	0.44	0.445
Q9NXF1	Testis-expressed protein 10	rRNA processing	0.441	0.418
P58004	Sestrin-2	Leucine sensor	0.441	0.181
Q9H993	Protein-glutamate O-methyltransferase	Enzyme	0.443	0.295
Q92618	Zinc finger protein 516	Transcriptional regulation	0.444	0.416
B4DJM5	cDNA FLJ61294, highly similar to Keratin, type I cytoskeletal 17	Pseudogene	0.446	0.344
Q9Y3B3	Transmembrane emp24 domain-containing protein 7	Intracellular transport	0.446	0.291
A0A024RAP4	Polymerase (DNA directed) kappa	DNA repair	0.448	0.248
B3KN76	cDNA FLJ13834 fis, clone THYRO1000684, highly similar to Homo sapiens MON1 homolog B	Uncharacterised	0.449	0.384

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q96N46	Tetratricopeptide repeat protein 14	RNA binding	0.449	0.403
Q8TAE6	Protein phosphatase 1 regulatory subunit 14C	Enzyme	0.45	0.298
Q53H54	tRNA wybutosine-synthesizing protein 2 homolog	tRNA modification	0.451	0.422
H7BY64	Uncharacterized protein	Uncharacterised	0.453	0.35
Q7L8W6	Diphthine-ammonia ligase	Enzyme	0.454	0.318
B3KQ21	cDNA FLJ32640, highly similar to U3 small nucleolar RNA-associated protein 6	rRNA processing	0.455	0.422
Q5JU23	Folylpolyglutamate synthase, mitochondrial	Enzyme	0.456	0.388
O15260	Surfeit locus protein 4	Golgi organisation	0.459	0.266
P10244	Myb-related protein B	Transcriptional regulation	0.459	0.433
P15924	Desmoplakin	Desmosome organisation, membrane protein	0.46	0.437
H0Y7R8	Afadin	Signal transduction, cell adhesion, membrane protein	0.46	0.437
Q2KHT3	Protein CLEC16A	Autophagy	0.462	0.407
O14949	Cytochrome b-c1 complex subunit 8	Respiratory chain	0.463	0.452
H0YAT7	Dystonin	Cytoskeletal linker protein	0.465	0.497
A0A1S6YJG6	Catechol O-methyltransferase isoform MB	Enzyme	0.465	0.28

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q6LC01	Tubulin beta chain	Component of cytoskeleton (microtubules)	0.467	0.379
Q53Y49	Forkhead box M1	Cell proliferation	0.467	0.318
A0A024RDB4	Heterogeneous nuclear ribonucleoprotei n D	RNA binding	0.468	0.32
Q15013	MAD2L1-binding protein	Cell cycle regulation	0.468	0.186
A0A024RAQ3	Dihydrofolate reductase	Enzyme	0.469	0.482
P41134	DNA-binding protein inhibitor ID-1	Transcriptional regulation	0.47	0.403
P55347	Homeobox protein PKNOX1	DNA binding	0.47	0.471
P08243	Asparagine synthetase	Enzyme	0.473	0.37
O60518	Ran-binding protein 6	Nuclear membrane transport, membrane protein	0.473	0.315
A0A140VJZ4	Ubiquitin carboxyl-terminal hydrolase	Enzyme	0.475	0.358
Q969Z3	Mitochondrial amidoxime reducing component 2	Mitochondrial protein	0.475	0.349
J3KPZ4	Nuclear nucleic acid-binding protein C1D	Apoptosis	0.475	0.455
Q9NRX1	RNA-binding protein PNO1	RNA binding	0.479	0.364
P13647	Keratin, type II cytoskeletal 5	Intermediate filament, cytoskeletal protein	0.48	0.443
A8K9U2	cDNA FLJ77474, highly similar to Homo sapiens ankyrin repeat and SOCS box-containing 6		0.481	0.448
Q9Y2X9	Zinc finger protein 281	Stem cell differentiation	0.484	0.408

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans*) / (VECs)	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q9NUJ1	Mycophenolic acid acyl-glucuronide esterase, mitochondrial	Enzyme	0.484	0.417
Q9BWT7	Caspase recruitment domain-containing protein 10	Apoptosis, activates NF-kappa-B	0.484	0.313
Q8WVD5	RING finger protein 141	Spermatogenesis	0.485	0.462
Q9H4L4	Sentrin-specific protease 3	Posttranslational modification of proteins	0.487	0.383
Q15746	Myosin light chain kinase, smooth muscle	Muscle contraction	0.489	0.468
Q5H9N4	Uncharacterized protein DKFZp686L20222	Uncharacterised	0.49	0.449
Q96EK4	THAP domain-containing protein 11	Transcriptional regulation	0.49	0.49
A0A087X0V5	2'-5'-oligoadenylate synthase 2	Antiviral response	0.491	0.427
O43572	A-kinase anchor protein 10, mitochondrial	Protein kinase A binding	0.493	0.44
A0A024R7M0	Transmembrane emp24 protein transport domain containing 9	Early secretory pathway	0.497	0.349

VEC proteins altered in expression in only the dual-species sample

Appendix 7 Overview of proteins that were upregulated in only the *C. albicans*+GBS-infected VECs relative to the VEC-only control.

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q8ND30	Liprin-beta-2	Cytoskeletal organisation	12.448
B2R932	cDNA, FLJ94187, highly similar to Homo sapiens CD99 antigen	Uncharacterised	6.259
Q9C0B0	RING finger protein unkempt homolog	Translational regulation	4.368
H7C2F2	CD99 antigen	Cytoskeletal organisation	4.059
B3KX23	cDNA FLJ44516 fis, highly similar to Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 42	Nucleic acid binding	3.802
Q86UE4	Protein LYRIC	Activates NF-kappa-B	3.585
F8WE98	Filamin-A	Cytoskeletal organisation	3.351
Q6ZUT6	Uncharacterized protein C15orf52	Uncharacterised	3.337
M0QZM1	Heterogeneous nuclear ribonucleoprotein M	RNA processing	3.26
Q7Z333	Probable helicase senataxin	RNA polymerase regulation	3.233
H0Y4V9	La-related protein 4B	Translational regulation	3.218
H0Y2V6	Centrosomal protein of 170 kDa	Cytoskeletal organisation of microtubules	3.127
P05089	Arginase-1	Regulator of immune responses	3.09
B4DW52	cDNA FLJ55253, highly similar to Actin	Uncharacterised	2.964
Q86UP2	Kinectin	Intracellular trafficking	2.908
F8W7C6	60S ribosomal protein L10	Ribosome subunit	2.859
Q96TA2	ATP-dependent zinc metalloprotease YME1L1	Protein degradation	2.848
P49711	Transcriptional repressor CTCF	MHC-II activator	2.83
A0A024R2V2	Microtubule-associated protein 4	Cytoskeletal organisation of microtubules	2.8

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
A0A1U9X8Y8	DNA-directed RNA polymerase subunit	RNA polymerase	2.798
G3V3T3	Apoptotic chromatin condensation inducer in the nucleus	Chromatin condensation	2.727
B4DN86	cDNA FLJ56047, highly similar to A kinase anchor protein 1, mitochondrial	RNA binding	2.716
Q86UE8	Serine/threonine-protein kinase tousled-like 2	Chromatin condensation	2.715
Q14686	Nuclear receptor coactivator 6	Activates NF-kappa-B	2.693
A0A024R926	Chromosome 1 open reading frame 21	Uncharacterised	2.649
Q8IUH3	RNA-binding protein 45	RNA binding	2.641
A0A024QZU8	Ras responsive element binding protein 1	Transcriptional regulation	2.609
A0A0R9RWK2	Erb-b2 receptor tyrosine kinase 2	Binds EGF receptor to stabilise ligand binding	2.607
Q9HD47	Ran guanine nucleotide release factor	Uncharacterised	2.603
B9VJ68	Toll-like receptor 5	TLR5	2.592
E9PR17	CD59 glycoprotein	Inhibits complement-mediated cell lysis	2.585
B4E2S3	cDNA FLJ56561	RNA binding	2.562
H7BZB9	Microtubule-associated protein 2	Cytoskeletal protein	2.547
A0A024RCN4	Zinc finger and SCAN domain-containing protein 26	Transcriptional regulation	2.525
P48426	Phosphatidylinositol 5-phosphate 4-kinase type-2 alpha	Autophagy regulation	2.518
E5RJF8	Centrin-3	Centrosome	2.517
Q9H270	Vacuolar protein sorting-associated protein 11 homolog	Intracellular trafficking	2.511
O60354	Loricrin	Uncharacterised	2.475

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
M0R0H3	mRNA decay activator protein ZFP36	Suppresses TNF-a	2.462
A0A024R046	High mobility group nucleosomal binding domain 4	Chromatin expansion	2.456
P07305	Histone H1.0	Histone	2.441
V9HW88	Calreticulin	Transcriptional regulation	2.434
A0A1B0GVU9	Glutamine-tRNA ligase	tRNA aminoacylation	2.428
B3KY59	cDNA FLJ46903 fis, clone MESAN2003661, highly similar to Cdc42 effector protein 3	Uncharacterised	2.426
K7ENT6	Tropomyosin alpha-4 chain	Cytoskeletal organisation of actin	2.42
G3V203	60S ribosomal protein L18	Ribosome subunit	2.414
A8MZ36	Envoplakin-like protein	Cytoskeletal organisation	2.41
B4DJQ5	cDNA FLJ59211, highly similar to Glucosidase 2 subunit beta	Calcium binding	2.404
A0A024RB53	Heterogeneous nuclear ribonucleoprotein A1	RNA processing	2.401
P16035	Metalloproteinase inhibitor 2	Metalloproteinase inhibitor	2.395
A3R0T8	Histone 1, H1e	Histone	2.394
A8K4E0	cDNA FLJ78122	Uncharacterised	2.393
Q13443	Disintegrin and metalloproteinase domain-containing protein 9	Cytoskeletal organisation, mediates cell motility, cell-cell and cell-matrix interactions	2.393
A0A0J9YXJ0	CUGBP Elav-like family member 2	RNA processing	2.379
P14625	Endoplasmin	Protein processing, Toll-like receptor folding	2.375
Q5VSQ6	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (Proline 4-	Collagen synthesis	2.362

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	hydroxylase), alpha polypeptide I variant		
Q96GD7	CSDA protein	Transcriptional regulation	2.359
Q53H37	Calmodulin-like skin protein variant	Uncharacterised	2.356
P35712	Transcription factor SOX-6	Transcriptional regulation	2.349
P09629	Homeobox protein Hox-B7	Transcription factor involved in cell proliferation	2.336
Q86YZ3	Hornerin	Keratinisation, component of epidermal cell envelope	2.324
Q14554	Protein disulfide-isomerase A5	Protein folding	2.324
P35222	Catenin beta-1	Transcriptional regulation, membrane bound, localises to adherens junctions and affects integrity of epithelial monolayer	2.319
A8K1R1	Receptor expression-enhancing protein	Uncharacterised	2.302
Q9NUD5	Zinc finger CCHC domain-containing protein 3	RNA binding	2.299
Q6UXH1	Cysteine-rich with EGF-like domain protein 2		2.299
O43818	U3 small nucleolar RNA-interacting protein 2	RNA processing	2.295
H0Y5B0	Band 4.1-like protein 2	Cytoskeletal organisation	2.285
Q59G46	Thioredoxin-like 1 variant	Cell redox homeostasis	2.283
V9GYF0	Rho guanine nucleotide exchange factor 2	Activates NF-kappa-B	2.276
P16403	Histone H1.2	Histone	2.265
Q7Z434	Mitochondrial antiviral-signaling protein	Innate immunity to viruses	2.265
Q5SXM2	snRNA-activating protein complex subunit 4	Transcriptional regulation	2.264

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q9Y3E1	Hepatoma-derived growth factor-related protein 3	DNA synthesis	2.261
O15516	Circadian locomoter output cycles protein kaput	Circadian clock	2.257
G3XAI2	Laminin subunit beta-1	ECM protein	2.251
F8W031	Uncharacterized protein	Uncharacterised	2.25
P28908	Tumor necrosis factor receptor superfamily member 8	TNF receptor, NF-kappa-B activation	2.242
Q06828	Fibromodulin	TGF-b receptor assembly	2.24
I3L0Y6	NmrA-like family domain-containing protein 1	Negatively regulates NF-kappa-B	2.236
P13645	Keratin, type I cytoskeletal 10	Cytoskeletal protein	2.232
P11047	Laminin subunit gamma-1	ECM protein	2.226
A7MBN3	Collagen, type IV, alpha 5	ECM protein	2.222
A8K3Y5	cDNA FLJ78186	Uncharacterised	2.217
H7BY36	RNA-binding protein EWS	RNA processing	2.215
Q96SB3	Neurabin-2	Cytoskeletal organisation of actin	2.213
D3DS14	Uncharacterized protein	Uncharacterised	2.208
P05165	Propionyl-CoA carboxylase alpha chain, mitochondrial	Citric acid cycle enzyme	2.199
A0A024R8S5	Protein disulfide-isomerase	Protein processing	2.196
B4E0X1	Beta-2-microglobulin	MHC class I component, involved in antigen presentation	2.191
G3V3F7	X-linked retinitis pigmentosa GTPase regulator-interacting protein 1	Uncharacterised	2.19
P30040	Endoplasmic reticulum resident protein 29	Protein folding	2.171

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
P54802	Alpha-N-acetylglucosaminidase	Degrades heparan sulphate	2.171
Q8N129	Protein canopy homolog 4	TLR4 regulation	2.155
A0A0S2Z4Z6	Serine/arginine repetitive matrix 1 isoform 2	RNA processing	2.151
K7EN83	EPS8-like 1	EGF receptor-like	2.144
A0A0G2JN70	Leukocyte receptor cluster member 8	Uncharacterised	2.124
Q9NYF3	Protein FAM53C	Transcriptional regulation	2.117
P13726	Tissue factor	Involved in cytokine receptor activity, membrane bound	2.115
Q5JW30	Double-stranded RNA-binding protein Staufen homolog 1	RNA transport	2.113
Q8WXD2	Secretogranin-3	Neuroendocrine secretory protein	2.113
Q9BRA0	N-alpha-acetyltransferase 38, NatC auxiliary subunit	Apoptosis repressor	2.111
Q86YL7	Podoplanin	Cytoskeletal organisation	2.107
Q9NX58	Cell growth-regulating nucleolar protein	RNA binding	2.105
Q6ZSJ8	Uncharacterized protein C1orf122	Uncharacterised	2.101
Q13092	Epidermal type I keratin	Cytoskeletal protein	2.095
P14316	Interferon regulatory factor 2	MHC class I repression, cell cycle regulation	2.091
P36954	DNA-directed RNA polymerase II subunit RPB9	RNA polymerase	2.09
Q92508	Piezo-type mechanosensitive ion channel component 1	Epithelial adhesion, membrane bound	2.089
Q86U75	Dihydropyrimidinase-like 2	Possible cytoskeletal organisation of microtubles	2.084
Q5D862	Filaggrin-2	Epithelial homeostasis	2.08

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
P62745	Rho-related GTP-binding protein RhoB	Intracellular trafficking, membrane bound	2.077
Q86X29	Lipolysis-stimulated lipoprotein receptor	Tight junction assembly	2.072
E7EQY1	Protein FAM136A	Uncharacterised	2.067
B7Z5R7	cDNA FLJ61355, highly similar to CLIP-associating protein 1	Possible cytoskeletal organisation of microtubules	2.063
O75712	Gap junction beta-3 protein	Gap junction, membrane bound	2.061
Q9BRL6	Serine/arginine-rich splicing factor 8	RNA binding	2.059
Q02447	Transcription factor Sp3	Transcriptional regulation	2.057
Q02880	DNA topoisomerase 2-beta	Topoisomerase	2.053
P81605	Dermcidin	Antimicrobial peptide	2.052
Q9NWM8	Peptidyl-prolyl cis-trans isomerase FKBP14	Protein folding	2.051
A8KAJ3	cDNA FLJ77823, highly similar to Homo sapiens EGF-containing fibulin-like extracellular matrix protein 1	EGF receptor-like	2.047
A0A087WZM2	Ribonuclease T2	RNA binding	2.044
P25942	Tumor necrosis factor receptor superfamily member 5	Mediates signalling from members of TNF receptor superfamily	2.043
A0A0S2Z4G4	Tropomyosin 3	Cytoskeletal organisation of actin	2.039
A8K6R0	cDNA FLJ75726, highly similar to Homo sapiens basic leucine zipper nuclear factor 1	Intracellular transport	2.036
B3KVV6	cDNA FLJ41607 fis, highly similar to Homo sapiens alpha-2-macroglobulin-like 1	Uncharacterised	2.035

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q96AY3	Peptidyl-prolyl cis-trans isomerase FKBP10	Protein folding	2.034
Q9NYU2	UDP-glucose:glycoprotein glucosyltransferase 1	Protein folding	2.03
P25398	40S ribosomal protein S12	Ribosome subunit	2.029
A0A024QZ72	PR domain containing 2, with ZNF domain	Transcriptional regulation	2.024
C9JTA2	Mitotic spindle assembly checkpoint protein MAD1	Cell cycle progression	2.022
A0A024R968	Calcium-transporting ATPase	ATP hydrolysis, calcium transport	2.021
O95218	Zinc finger Ran-binding domain-containing protein 2	RNA processing	2.02
A0A024QYW3	Proteolipid protein 2 (Colonic epithelium-enriched)	Ion channel	2.016
P08572	Collagen alpha-2(IV) chain	ECM protein	2.014

Appendix 8 Overview of proteins that were downregulated in only the *C. albicans*+GBS-infected VECs relative to the VEC-only control.

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
B1PS43	Myosin heavy chain 11 smooth muscle isoform	Uncharacterised	0.01
P14373	Zinc finger protein RFP	Ubiquitination, induces apoptosis	0.01
B2R533	cDNA, FLJ92320, highly similar to Homo sapiens glutathione S-transferase theta 2	Transferase	0.204

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q9Y679	Ancient ubiquitous protein 1	Protein degradation	0.243
O76095	Protein JTB	Cell cycle progression, may inhibit apoptosis induced by TGFB1	0.273
P36542	ATP synthase subunit gamma	ATP Synthase, membrane bound	0.276
O43615	Mitochondrial import inner membrane translocase subunit TIM44	Mitochondrial protein	0.292
P51668	Ubiquitin-conjugating enzyme E2 D1	Ubiquitination	0.293
O95470	Sphingosine-1-phosphate lyase 1	Fatty acid enzyme, involved in apoptosis signalling	0.294
Q14019	Coactosin-like protein	Actin binding	0.296
A0A140VJX5	Testicular tissue protein Li 202	Uncharacterised	0.297
T2C6S4	WWC family member 3	Uncharacterised	0.306
P53677	AP-3 complex subunit mu-2	Intracellular protein transport	0.312
Q8NI62	Ribosomal protein S2	Ribosomal formation	0.315
P49411	Elongation factor Tu, mitochondrial	Elongation factor	0.318
D7UNW5	Polypeptide N-acetylgalactosaminyltransferase	Protein O-linked glycosylation	0.323
P36952	Serpin B5	Tumour supressor	0.328
P82650	28S ribosomal protein S22, mitochondrial	Mitochondrial ribosome	0.33
O60287	Nucleolar pre-ribosomal-associated protein 1	Uncharacterised	0.333
P50583	Bis(5'-nucleosyl)-tetraphosphatase	Tumour supressor	0.338
D3YTI2	Low molecular weight phosphotyrosine protein phosphatase	Uncharacterised	0.339
Q8TEP9	FLJ00144 protein	Uncharacterised	0.341

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q9H4A4	Aminopeptidase B	Uncharacterised	0.342
Q9NTX5	Ethylmalonyl-CoA decarboxylase	Uncharacterised	0.343
B2RAH7	cDNA, FLJ94921, highly similar to Homo sapiens prolyl endopeptidase	Uncharacterised	0.344
X6RAY8	39S ribosomal protein L4, mitochondrial	Mitochondrial ribosome	0.347
O75964	ATP synthase subunit g, mitochondrial	ATP Synthase	0.348
F5H4M0	Vacuolar protein sorting-associated protein 37B	Uncharacterised	0.349
Q8TBM8	DnaJ homolog subfamily B member 14	Protein trafficking	0.349
A8K710	Sterile alpha motif and leucine zipper containing kinase AZK	MAPK pathway, pro-apoptotic activity	0.353
Q8WUX2	Putative glutathione-specific gamma-glutamylcyclotransferase 2	Catalytic enzyme	0.353
Q96KB5	Lymphokine-activated killer T-cell-originated protein kinase	MAPK pathway	0.354
P45954	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	Fatty acid enzyme	0.356
H7C1N3	BET1 homolog	Golgi-to-ER transport	0.356
Q8WZ82	Esterase OVCA2	Uncharacterised	0.359
P16152	Carbonyl reductase [NADPH]	Reductase	0.361
O00762	E2 C	Ubiquitin-conjugating enzyme	0.361
Q86TI0	TBC1 domain family member 1	Intracellular protein transport	0.361
P49406	39S ribosomal protein L19, mitochondrial	Mitochondrial ribosome	0.363

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
A0A140VK27	Leukotriene A(4) hydrolase	Degrades neutrophil chemoattractant	0.364
A0A024RC48	Polypeptide N-acetylgalactosaminyltransferase	Protein O-linked glycosylation	0.364
Q9Y2S2	Lambda-crystallin homolog	Fatty acid enzyme	0.364
Q9H1A3	Methyltransferase-like protein 9	Uncharacterised	0.365
A8K3B6	Tyrosine-protein kinase	Suppresses TCR and BCR signalling	0.367
P19623	Spermidine synthase	Catalytic enzyme	0.368
Q9Y2L6	FERM domain-containing protein 4B	Epithelial cell polarity	0.368
Q5VWZ2	Lysophospholipase-like protein 1	Protein depalmitoylation	0.372
X6R2S6	Signal peptidase complex subunit 1	Peptide processing	0.373
B4DZK0	Cysteine protease	Protein trafficking	0.374
Q969T4	Ubiquitin-conjugating enzyme E2 E3	Ubiquitination	0.374
A0A087WXX8	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	Respiratory enzyme	0.375
Q53F60	Carbonyl reductase 3 variant	Reductase	0.376
P51965	Ubiquitin-conjugating enzyme E2 E1	Catalytic enzyme	0.378
P67812	Signal peptidase complex catalytic subunit SEC11A	Signal transduction, membrane bound	0.38
Q2Q9H2	Glucose-6-phosphate 1-dehydrogenase	Non-glycolytic enzyme	0.381
Q9HAB8	Phosphopantothenate--cysteine ligase	Catalytic enzyme	0.383
B4DH89	cDNA FLJ55560, highly similar to Retinal dehydrogenase 2	Catalytic enzyme	0.383

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q16763	Ubiquitin-conjugating enzyme E2 S	Ubiquitination	0.383
Q5BKZ8	HSPA12B protein	Heat stress response, cell senescence	0.383
B3KM58	cDNA FLJ10358 fis, highly similar to Glutaminase kidney isoform, mitochondrial	Catalytic enzyme	0.384
Q96AB6	Protein N-terminal asparagine amidohydrolase	Hydrolase	0.386
L7RSM2	Mitogen-activated protein kinase	MAPK pathway, activated by proinflammatory cytokines	0.387
P46977	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit STT3A	Protein modification	0.387
A8K7T4	cDNA FLJ75774, highly similar to Homo sapiens lectin, mannose-binding 2	Protein trafficking	0.389
P61086	Ubiquitin-conjugating enzyme E2 K	Ubiquitin conjugating enzyme, involved in processing of NF-kappa-B	0.39
Q9HCC0	Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	Mitochondrial enzyme	0.393
B3KN28	Phosphoacetylglucosamine mutase	Glycosylation	0.395
B3KY29	Poly [ADP-ribose] polymerase	Cell cycle progression	0.396
Q00013	55 kDa erythrocyte membrane protein	Signal transduction, membrane bound	0.396
Q6PCE3	Glucose 1,6-bisphosphate synthase	Catalytic enzyme	0.397
A8K7F6	cDNA FLJ78244, highly similar to Homo sapiens eukaryotic	Uncharacterised	0.399

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	translation initiation factor 4A		
Q9BUK6	Protein misato homolog 1	Mitochondrial morphology	0.399
B2R4D5	Actin-related protein 2/3 complex subunit 3	Cytoskeletal organisation of actin	0.399
A4D1L5	Ubiquitin-conjugating enzyme E2H	Ubiquitination	0.404
Q9NPB8	Glycerophosphocholine phosphodiesterase GPCPD1	Uncharacterised	0.405
Q9Y2S6	Translation machinery-associated protein 7	Uncharacterised	0.405
A0A024QZN9	Voltage-dependent anion channel 2	Apoptosis induction	0.406
E9KL44	Epididymis tissue sperm binding protein Li 14m	Mitochondrial enzyme	0.406
B3KRI8	cDNA FLJ34373 fis, highly similar to Dual specificity protein kinase CLK3	Protein kinase	0.406
E7EQR8	Protein YIPF3	Uncharacterised	0.406
B4DHQ3	Phosphoserine aminotransferase	Uncharacterised	0.407
Q9UJU1	Cytovillin 2	Possible cytoskeletal protein binding	0.407
O95372	Acyl-protein thioesterase 2	Fatty acid enzyme	0.407
B7WPL0	Synembryn-B	Uncharacterised	0.407
Q16539	Mitogen-activated protein kinase 14	MAPK pathway, activated by proinflammatory cytokines	0.409
Q53ET9	Ariadne homolog 2 variant	Protein degradation	0.409
Q9H2W6	39S ribosomal protein L46, mitochondrial	Mitochondrial ribosome	0.409
Q4LDG9	Dynein light chain 1, axonemal	Dynein	0.409
P07741	Adenine phosphoribosyltransferase	AMP salvage	0.41

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q96K37	Solute carrier family 35 member E1	Uncharacterised	0.411
A0A024R172	Leukotriene B4 12-hydroxydehydrogenase	Chemoattractant leukotriene B4 inactivation	0.414
O75884	Putative hydrolase RBBP9	Uncharacterised	0.414
P42574	Caspase-3	Activation of caspases responsible for apoptosis	0.415
Q53GP2	Thioredoxin-like 4B variant	Cell cycle progression	0.416
Q6ZMR3	L-lactate dehydrogenase A-like 6A	LDH	0.417
Q96LR5	Ubiquitin-conjugating enzyme E2 E2	Ubiquitination	0.418
Q59G70	Mannosyl (Alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase variant	Glycosylation	0.418
A0A024QZN6	Retinoic acid induced 17	Regulates transcription factors	0.418
B4E2Q0	Calcium-transporting ATPase	ATPase	0.419
B2RE46	cDNA, FLJ96923, highly similar to Homo sapiens ribophorin II	N-linked glycosylation	0.42
A0A024R718	Pre-B-cell colony enhancing factor 1	Circadian clock (& anti-diabetic cytokine)	0.421
D6RFG8	Deoxycytidine kinase	Kinase	0.421
B4DDK9	cDNA FLJ55952, highly similar to Alpha-1,6-mannosyl-glycoprotein2-beta-N-acetylglucosaminyltransferase	Catalytic enzyme	0.421
Q96BX8	MOB kinase activator 3A	Uncharacterised	0.421
J3KQE5	GTP-binding nuclear protein Ran	Nucleocytoplasmic transport	0.423

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
B2R9H3	cDNA, FLJ94391, highly similar to Homo sapiens serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 8	Uncharacterised	0.423
A0A024R2A7	Lectin, mannose-binding, 1	Glycoprotein transport	0.423
Q59ET0	Glucan, branching enzyme 1 variant	Glycogen branching	0.423
Q9NRL2	Bromodomain adjacent to zinc finger domain protein 1A	Chromatin remodelling	0.423
B3KQQ9	cDNA PSEC0048 fis, highly similar to Serine protease 23	Uncharacterised	0.423
Q96GX2	Putative ataxin-7-like protein 3B	Ubiquitination	0.425
Q9BS40	Latexin	Inflammatory response	0.426
Q6ZWE6	Pleckstrin homology domain-containing family M member 3	Uncharacterised	0.427
V9HWH1	Epididymis luminal protein 57	Protease inhibitor	0.428
F8VZY9	Keratin, type I cytoskeletal 18	Cytoskeletal protein	0.429
Q9Y217	Myotubularin-related protein 6	Phosphatase, regulates CD4 T cell proliferation	0.429
Q9NYK5	39S ribosomal protein L39, mitochondrial	Mitochondrial ribosome	0.429
A0A024QYR3	Transmembrane 9 superfamily member	Protein trafficking	0.43
A0A0A0MRP2	Fucose-1-phosphate guanylyltransferase	Uncharacterised	0.43
Q5JPI9	EEF1A lysine methyltransferase 2	Catalytic enzyme	0.43
P30520	Adenylosuccinate synthetase isozyme 2	Catalytic enzyme	0.431
B2R7T6	cDNA, FLJ93596, highly similar to Homo sapiens	Mitochondrial enzyme	0.431

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	sulfide quinone reductase-like		
O75381	Peroxisomal membrane protein PEX14	Intracellular protein transport, membrane bound	0.431
Q9NS18	Glutaredoxin-2, mitochondrial	Mitochondrial enzyme	0.432
B4DQE1	Annexin	Uncharacterised	0.433
Q8IY81	pre-rRNA processing protein FTSJ3	Ribosomal formation	0.433
O95861	3'(2'),5'-bisphosphate nucleotidase 1	Catalytic enzyme	0.433
J3KQY1	39S ribosomal protein L22, mitochondrial	Mitochondrial ribosome	0.433
A0A0J9YXC7	LIM and senescent cell antigen-like-containing domain protein	Links integrins to the actin cytoskeleton	0.434
Q53GF9	Full-length cDNA 5-PRIME end of clone CS0DF013YM24 of Fetal brain	Intracellular protein transport	0.434
Q96K80	Zinc finger CCCH domain-containing protein 10	Regulates gene expression	0.434
Q16222	UDP-N-acetylhexosamine pyrophosphorylase	Catalytic enzyme	0.435
P52895	Aldo-keto reductase family 1 member C2	Reductase	0.435
Q9UKM7	Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase	Protein degradation	0.435
Q8WVY7	Ubiquitin-like domain-containing CTD phosphatase 1	Phosphatase	0.436
Q96BN8	Ubiquitin thioesterase otulin	Regulates innate immune response to limit proinflammatory signalling, negative regulation of NF-kappa-B	0.436
Q96CF2	Charged multivesicular body protein 4c	Intracellular protein transport	0.436

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
A2RUC4	tRNA wybutosine-synthesizing protein 5	tRNA hydroxylase	0.436
Q9NQZ5	StAR-related lipid transfer protein 7, mitochondrial	Uncharacterised	0.436
B3KSH8	cDNA FLJ36241 fis, highly similar to Inositol polyphosphate 1-phosphatase	Phosphatidylinositol signaling pathway	0.437
B3KVH4	cDNA FLJ16549 fis, highly similar to RAC-alpha serine/threonine-protein kinase	AKT kinase	0.437
K7EQZ3	Uncharacterized protein	Uncharacterised	0.438
A8K9G4	FLJ77745	Regulates TNF-a	0.439
A0A024R782	Phosphoglycerate mutase	Glycolytic process	0.439
Q9H668	CST complex subunit STN1	Regulates DNA replication	0.439
B4DQK8	cDNA FLJ54750, moderately similar to Pyrroline-5-carboxylate reductase 2	Uncharacterised	0.44
B4DI08	cDNA FLJ60091, highly similar to Hypoxia-inducible factor 1 alpha inhibitor	Oxygen sensor	0.441
V9HWJ2	Isocitrate dehydrogenase [NADP]	Mitochondrial enzyme	0.442
A0A0A0MQW3	Serpin B13	Keratinocyte proliferation and differentiation	0.442
Q01415	N-acetylgalactosamine kinase	Kinase	0.442
Q9HC07	Transmembrane protein 165	Calcium transporter	0.443
Q01954	Zinc finger protein basonuclin-1	Cell differentiation	0.444
B2R739	cDNA, FLJ93269, highly similar to	Mitochondrial ribosome	0.445

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	Homo sapiens mitochondrial ribosomal protein L15, nuclear gene encoding mitochondrial protein		
Q9BV61	TRAP1 protein	Mitochondrial chaperone	0.447
Q9P287	BRCA2 and CDKN1A-interacting protein	Cytoskeletal organisation of microtubles	0.447
B5BUI8	Dual specificity phosphatase 3	Phosphatase	0.447
J3KQ18	D-dopachrome decarboxylase	Decarboxylase factor	0.447
P21266	Glutathione S-transferase Mu 3	Glutathione transferase	0.448
Q9Y570	Protein phosphatase methylesterase 1	Protein demethylation	0.448
A0A024RA66	Cell division cycle 2-like 5 (Cholinesterase-related cell division controller)	Regulates transcriptional elongation	0.448
A0A024RDJ1	DC2 protein	Uncharacterised	0.448
Q15819	Ubiquitin-conjugating enzyme E2 variant 2	Ubiquitin conjugating enzyme	0.449
O43708	Maleylacetoacetate isomerase	Catalytic enzyme	0.449
A8K8D9	Glucose-6-phosphate 1-dehydrogenase	Non-glycolytic enzyme	0.45
Q6PIW8	COG4 protein	Golgi protein	0.45
Q9H992	E3 ubiquitin-protein ligase MARCH7	Ubiquitin ligase	0.451
D3DPA6	WD repeat, sterile alpha motif and U-box domain containing 1	Uncharacterised	0.453
Q6FHU2	Phosphoglycerate mutase	Glycolysis enzyme	0.454
Q9NR19	Acetyl-coenzyme A synthetase, cytoplasmic	Synthetase	0.454
Q6N0B1	Succinate-CoA ligase subunit beta	Mitochondrial enzyme	0.454

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
D6RB97	Rap1 GTPase-GDP dissociation stimulator 1	GTPase	0.454
Q13907	Isopentenyl-diphosphate Delta-isomerase 1	Catalytic enzyme	0.455
A0A024R9A9	Ubiquitin-conjugating enzyme E2T	Ubiquitination	0.455
Q9BU02	Thiamine-triphosphatase	Catalytic enzyme	0.455
P68366	Tubulin alpha-4A chain	Cytoskeletal protein	0.456
V9HW91	Epididymis secretory sperm binding protein Li 8a	Uncharacterised	0.456
Q9UK99	F-box only protein 3	Protein degradation	0.457
A0A024QYX0	Emopamil binding protein	Transmembrane signalling receptor activity	0.457
A0A024R012	NAD-dependent protein deacylase sirtuin-5, mitochondrial	Deacylase	0.458
A0A024RDY0	RAN binding protein 5	Intracellular protein transport	0.459
P21399	Cytoplasmic aconitate hydratase	RNA binding	0.459
A0A1L7NY50	Polypeptide N-acetylgalactosaminyltransferase	Glycosyltransferase, glycosylates hinge region of IgA	0.459
Q16822	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	Mitochondrial enzyme	0.459
Q15797	Mothers against decapentaplegic homolog 1	Receptor kinase	0.459
Q9H4B6	Protein salvador homolog 1	Promotes apoptosis	0.459
P06737	Glycogen phosphorylase, liver form	Glycogen phosphorylase	0.46
A8K669	FLJ78452	Peptide processing for MHC class II	0.46
P55212	Caspase-6	Caspase, overexpression leads to apoptosis	0.46

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
A0A024RAD5	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit	Glycosyltransferase	0.46
Q9NUQ7	Ufm1-specific protease 2	Protease	0.46
Q5VW32	BRO1 domain-containing protein BROX	Uncharacterised	0.461
Q9UHY7	Enolase-phosphatase E1	Phosphatase	0.462
A0A0A0MSS8	Aldo-keto reductase family 1 member C3	Catalytic enzyme	0.462
B0I1P6	Kidney ankyrin repeat-containing protein 4	Cytoskeletal organisation of actin	0.462
A0A024RC24	Impact homolog	Stress response	0.463
P11216	Glycogen phosphorylase, brain form	Glycogen phosphorylase	0.464
B2R7G6	cDNA, FLJ93437, highly similar to Homo sapiens histidyl-tRNA synthetase-like	Moderates protein biosynthesis	0.464
Q8NEC7	Glutathione S-transferase C-terminal domain-containing protein	Uncharacterised	0.464
O00483	Cytochrome c oxidase subunit NDUFA4	Mitochondrial enzyme	0.464
Q9NS00	Glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1	Glycosyltransferase	0.464
Q6ICB0	Desumoylating isopeptidase 1	Protease	0.464
P52306	Rap1 GTPase-GDP dissociation stimulator 1	GTPase	0.465
P04843	Dolichyl-diphosphooligosaccharide-protein	Catalytic enzyme, membrane bound	0.465

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	glycosyltransferase subunit 1		
A0A024R687	Pleckstrin homology domain containing, family C (With FERM domain) member 1	Cytoskeletal organisation of actin, interacts with integrin	0.465
O00154	Cytosolic acyl coenzyme A thioester hydrolase	Fatty acid enzyme	0.466
H3BU16	Jupiter microtubule-associated homolog 2	Uncharacterised	0.466
G3V5T9	Cyclin-dependent kinase 2	Cell cycle progression	0.466
Q5T7V8	RAB6-interacting golgin	Uncharacterised	0.466
Q6IAQ2	SDHC protein	Mitochondrial enzyme	0.466
Q9BTV5	Fibronectin type III and SPRY domain-containing protein 1	Cytoskeletal organisation of microtubules	0.466
Q86V88	Magnesium-dependent phosphatase 1	Phosphatase	0.467
Q53EP0	Fibronectin type III domain-containing protein 3B	Uncharacterised	0.467
P0CG31	Putative zinc finger protein 286B	Uncharacterised	0.468
P12429	Annexin A3	Inhibitor of phospholipase	0.469
B2RE94	EEF1A lysine methyltransferase 1	Uncharacterised	0.469
Q5SY16	Polynucleotide 5'-hydroxyl-kinase NOL9	rRNA processing	0.47
Q86WQ0	Nuclear receptor 2C2-associated protein	Uncharacterised	0.47
A0A024R275	Riboflavin kinase	TNF-α pathway	0.47
Q8TE77	Protein phosphatase Slingshot homolog 3	Cytoskeletal organisation of actin filaments	0.471
B8ZZC8	Methyltransferase-like protein 5	Uncharacterised	0.471
Q9UIL1	Short coiled-coil protein	Golgi-to-ER transport	0.471
Q96A26	Protein FAM162A	Possibly regulates apoptosis	0.471

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q02750	Dual specificity mitogen-activated protein kinase kinase 1	MAPK pathway	0.476
Q9P0T4	Zinc finger protein 581	Uncharacterised	0.476
B4DV48	cDNA FLJ55526, highly similar to Complement C2	Uncharacterised	0.476
A0A024R4U3	Tubulin tyrosine ligase-like family, member 12	Uncharacterised	0.477
P30043	Flavin reductase (NADPH)	Reductase	0.477
Q9BYE7	Polycomb group RING finger protein 6	Transcriptional repressor	0.477
A0A0S2Z3H1	Caspase 2 apoptosis-related cysteine peptidase	Caspase	0.477
A0A0K0K1K7	6-phosphogluconolactonase	Catalytic enzyme	0.478
A0A0S2Z542	Mitogen-activated protein kinase	MAPK pathway, activated by proinflammatory cytokines	0.479
Q13951	Core-binding factor subunit beta	DNA binding	0.479
J3QL71	Secernin-2	Uncharacterised	0.479
A0A024RAN2	Calpastatin	Membrane fusion	0.48
Q06124	Tyrosine-protein phosphatase non-receptor type 11	Cell signalling	0.48
Q9BVP2	Guanine nucleotide-binding protein-like 3	Uncharacterised	0.48
Q9Y4W2	Ribosomal biogenesis protein LAS1L	Ribosomal formation	0.48
B4DP80	NAD(P)H-hydrate epimerase	Catalytic enzyme	0.48
A0A087WZT3	BolA-like protein 2	Iron maturation	0.48
Q9UNL2	Translocon-associated protein subunit gamma	Protein transport	0.48
Q9NVP2	Histone chaperone ASF1B	Chromatin remodelling	0.48

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
P04275	von Willebrand factor	Von Willebrand Factor	0.48
I3L3Q4	Glyoxalase domain-containing protein 4	Uncharacterised	0.481
P31751	RAC-beta serine/threonine-protein kinase	AKT kinase, regulates NF-kappa-B	0.481
A0A0G2JH58	MHC class I polypeptide-related sequence B	MHC class I pathway	0.481
F5H5D3	Tubulin alpha chain	Cytoskeletal protein	0.482
Q5U077	L-lactate dehydrogenase	LDH	0.483
P07737	Profilin-1	Cytoskeletal organisation of actin	0.483
Q07960	Rho GTPase-activating protein 1	GTPase	0.483
P27449	V-type proton ATPase 16 kDa proteolipid subunit	Proton transport, membrane bound	0.483
V9HWC7	Epididymis secretory sperm binding protein Li 128m	Fatty acid enzyme	0.484
Q96S55	ATPase WRNIP1	Moderates DNA Polymerase	0.484
P00338	L-lactate dehydrogenase A chain	LDH	0.485
P10586	Receptor-type tyrosine-protein phosphatase F	Cell adhesion receptor	0.485
A0A024R3Z6	Basic leucine zipper and W2 domains 1	Uncharacterised	0.486
Q9NRN7	L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	Post-translational modification	0.486
Q96CN7	Isochorismatase domain-containing protein 1	Uncharacterised	0.486
A0A024R398	Ankyrin repeat domain 49	NF-kappa-B pathway	0.486
A0A140VJT8	Testicular tissue protein Li 164	RNase inhibitor	0.487

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
Q14512	Fibroblast growth factor-binding protein 1	Fibroblast growth factor binding	0.487
Q7Z589	BRCA2-interacting transcriptional repressor EMSY	Transcription factor, DNA repair	0.487
B4DZS0	DNA fragmentation factor subunit beta	DNase	0.487
P14550	Alcohol dehydrogenase [NADP(+)]	Catalytic enzyme	0.488
P31939	Bifunctional purine biosynthesis protein PURH	Insulin receptor promotion	0.489
Q9NP77	RNA polymerase II subunit A C-terminal domain phosphatase SSU72	RNA polymerase	0.489
A8K2P7	cDNA FLJ77652, highly similar to Homo sapiens START domain containing 4, sterol regulated	Uncharacterised	0.489
P55786	Puromycin-sensitive aminopeptidase	Antigen processing for MHC class I	0.49
O95999	B-cell lymphoma/leukemia 10	Adaptive immune response, promotes apoptosis and activation of NF-kappa-B	0.49
A0A024R1U5	Phosphomannomutase	Glycosylation	0.49
B4DKM0	cDNA FLJ51883, highly similar to Mitochondrial 39S ribosomal protein L3	Mitochondrial ribosome	0.49
J3KQ32	Obg-like ATPase 1	ATP Hydrolase	0.491
B4DQY2	MICOS complex subunit MIC60	Mitochondrial membrane protein	0.491
B2R7E8	cDNA, FLJ93412, highly similar to Homo sapiens replication protein A2	Stabilises DNA intermediates	0.491
Q92547	DNA topoisomerase 2-binding protein 1	DNA replication	0.491

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
P05783	Keratin, type I cytoskeletal 18	Cytoskeletal keratin	0.492
Q59ER5	WD repeat-containing protein 1 isoform 1 variant	Uncharacterised	0.492
A0A024R1I5	SEC14-like 2	Cholesterol biosynthesis pathway	0.492
Q8TDQ7	Glucosamine-6-phosphate isomerase 2	Phosphatase	0.492
Q9NZN3	EH domain-containing protein 3	Cytoskeletal organisation of microtubles	0.492
Q9BRA2	Thioredoxin domain-containing protein 17	Modulates TNF-α and NF-kappa-B activation	0.493
M0R398	Zinc finger protein 414	Uncharacterised	0.493
A0A024RBL2	Serine dehydratase-like	Uncharacterised	0.493
Q6AWC2	Protein WWC2	Regulates transcription	0.493
Q8NCY6	Myb/SANT-like DNA-binding domain-containing protein 4	Uncharacterised	0.493
A0A087X1U6	Epiplakin	Cytoskeletal organisation of keratin	0.494
P55084	Trifunctional enzyme subunit beta, mitochondrial	Catalytic enzyme	0.495
P27144	Adenylate kinase 4, mitochondrial	Catalytic enzyme	0.495
H7BYJ1	E3 ubiquitin-protein ligase RNF34	Ubiquitin ligase, resists TNF-α mediated apoptosis	0.495
E9PJ55	T-complex protein 11-like protein 1	Uncharacterised	0.495
P55263	Adenosine kinase	Adenosine kinase	0.496
P52735	Guanine nucleotide exchange factor VAV2	Guanine nucleotide exchange factor	0.496
B2RWN5	HEAT repeat containing 1	Ribosomal formation, processes rRNA	0.497
Q9NR31	GTP-binding protein SAR1a	Intracellular protein transport	0.497
Q8WU10	Pyridine nucleotide-disulfide oxidoreductase	Stress response	0.497

Protein code	Protein name	Protein function	Relative abundance (VECs + *C. albicans* + GBS) / (VECs)
	domain-containing protein 1		
B3KW34	Protein YIPF	Golgi-to-ER transport	0.497
Q3ZAQ7	Vacuolar ATPase assembly integral membrane protein VMA21	Regulates ATPase activity	0.497
O00148	ATP-dependent RNA helicase DDX39A	RNA helicase	0.499
P61081	NEDD8-conjugating enzyme Ubc12	Catalytic enzyme	0.499
O60499	Syntaxin-10	Intracellular protein transport, membrane bound	0.499

Appendix B: Published manuscript

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MOLECULAR PATHOGENESIS

Coassociation between Group B *Streptococcus* and *Candida albicans* Promotes Interactions with Vaginal Epithelium

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ABSTRACT Group B *Streptococcus* (GBS) is a leading cause of neonatal sepsis, pneumonia, and meningitis worldwide. In the majority of cases, GBS is transmitted vertically from mother to neonate, making maternal vaginal colonization a key risk factor for neonatal disease. The fungus *Candida albicans* is an opportunistic pathogen of the female genitourinary tract and the causative agent of vaginal thrush. Carriage of *C. albicans* has been shown to be an independent risk factor for vaginal colonization by GBS. However, the nature of interactions between these two microbes is poorly understood. This study provides evidence of a reciprocal, synergistic interplay between GBS and *C. albicans* that may serve to promote their cocolonization of the vaginal mucosa. GBS strains NEM316 (serotype III) and 515 (serotype Ia) are shown to physically interact with *C. albicans*, with the bacteria exhibiting tropism for candidal hyphal filaments. This interaction enhances association levels of both microbes with the vaginal epithelial cell line VK2/E6E7. The ability of GBS to coassociate with *C. albicans* is dependent upon expression of the hypha-specific adhesin Als3. In turn, expression of GBS antigen I/II family adhesins (Bsp polypeptides) facilitates this coassociation and confers upon surrogate *Lactococcus lactis* the capacity to exhibit enhanced interactions with *C. albicans* on vaginal epithelium. As genitourinary tract colonization is an essential first step in the pathogenesis of GBS and *C. albicans*, the coassociation mechanism reported here may have important implications for the risk of disease involving both of these pathogens.

KEYWORDS *Candida albicans*, *Streptococcus agalactiae*, adhesins, cocolonization, polymicrobial interactions, vaginal epithelium

Streptococcus agalactiae (group B *Streptococcus* [GBS]) is a leading cause of invasive disease (sepsis, pneumonia, and meningitis) in neonates and is responsible for life-threatening infections in elderly and immunocompromised individuals (1–3). GBS is an opportunistic pathogen of the female genitourinary (GU) tract, with a carriage rate in Western countries of approximately 30% (2). The primary route of transmission to neonates is from the mother during or preceding birth, with estimated transmission rates of up to 50% (2). Among neonates that are colonized, about 1% develop severe GBS disease, resulting in significant infant morbidity or mortality (2, 4).

A variety of proteins that may promote colonization of host mucosae have been identified on the surface of GBS. These include pili (5), alpha C protein (6), BibA (7), serine-rich repeat proteins (Srr1/2) (5, 8), FbsA (9), Lmb (10), and the recently characterized antigen I/II (AgI/II) family proteins, designated BspA to -D (11, 12). Many of these surface proteins have been shown to target receptors expressed directly on the cervical or vaginal epithelia, while others bind extracellular matrix (ECM) proteins, such as collagen, fibrinogen, fibronectin, or laminin (5, 8–10). An additional colonization strategy for GBS, but one that remains poorly understood, is via interactions with other members of the vaginal microbiota. It is widely accepted that a "healthy" vaginal microbiota is dominated (ca. 70%) by the genus *Lactobacillus*, but Gram-positive bacteria (e.g., streptococci and staphylococci), Gram-negative bacteria (e.g., *Escherichia coli*), and

yeasts (e.g., *Candida albicans*) are also frequently isolated (13). Of particular relevance to GBS colonization is a growing body of evidence indicating an association with the fungus *C. albicans*. In both developed and developing countries, vaginal carriage of *C. albicans* has been shown to be an independent risk factor for vaginal colonization by GBS (14–18).

C. albicans accounts for the fourth highest rate of systemic nosocomial infection in the United States (19), and as an opportunistic pathogen of the oropharynx and the female GU tract, it is the predominant cause of both oral and vaginal thrush. Key risk factors for *C. albicans* infection are immunosuppression, use of oral contraceptives, hormone therapy, antibiotics, diabetes, and pregnancy (20). A number of colonization determinants have been implicated in promoting candidal adhesion to and invasion of mucosae. These include proteins that are expressed on the surfaces of both morphological forms (blastospore and hypha) of *C. albicans*, such as Als1, Eap1, Eno1, Pra1, and Tdh1 (21–25). Other major candidal adhesins, including Hwp1, Als3, and Ssa1, are expressed exclusively on the filamentous hyphae (22, 26, 27). Similar to the case with GBS, epithelial receptor molecules (e.g., CEACAMs and cadherins) and ECM proteins (e.g., fibronectin and laminin) have been identified as targets of these *C. albicans* adhesins (25, 27–29).

Synergistic polymicrobial interactions have already been described for *C. albicans* and a number of Gram-positive bacteria. For example, the oral bacterium *Streptococcus gordonii* produces nutrient by-products that are stimulatory to *C. albicans*, enhancing the length of hyphal filaments (30). In turn, *S. gordonii* benefits from the reduced oxygen environment generated by *C. albicans* metabolism (31). Physical coadhesion of these two microbes also serves to promote retention of *C. albicans* within the oral cavity; the molecular basis of this was identified as recognition of the *C. albicans* adhesin Als3 by the *S. gordonii* AgI/II family protein SspB (32). Similar interactions have been reported for *Streptococcus mutans* and *C. albicans*, for which the *S. mutans* glucosyltransferase GtfB has been shown to bind mannans on the candidal cell surface, promoting robust cross-kingdom biofilm formation within the oral cavity of rats (33). In addition to niche colonization, interkingdom interactions may modulate disease progression. *Streptococcus oralis* and *C. albicans* synergize within the oropharynx to promote breakdown of epithelial tight junctions, resulting in enhanced systemic dissemination of *C. albicans* (34, 35). Likewise, *Staphylococcus aureus* has a high affinity for binding *C. albicans* hyphae and can “piggyback” on these filamentous forms as they infiltrate host cells to gain access to deeper tissues (36). Again, staphylococcal recognition of the *C. albicans* hyphal protein Als3 is critical for this coadhesion (37).

We recently characterized an AgI/II family polypeptide of GBS designated BspA. Alongside binding to the salivary pellicle and vaginal epithelium, BspA was shown to promote coaggregation of GBS strain NEM316 with *C. albicans* under planktonic conditions (12). The present study therefore aimed to build on these initial observations, determine in more detail the interkingdom interactions between GBS and *C. albicans*, and investigate the potential of these interactions to modulate the colonization or pathogenic capabilities of these two microbes within the GU tract.

RESULTS

Planktonic interactions of GBS and *C. albicans*. The first step in exploring the interactions of GBS and *C. albicans* was to confirm their capacity to coaggregate under planktonic conditions. The following two strains of GBS that represent two of the most common capsular serotypes associated with neonatal disease were tested: GBS strain 515 (capsular serotype Ia) and strain NEM316 (capsular serotype III) (Table 1). *C. albicans* was fluorescently labeled with calcofluor white, while GBS strains were labeled with fluorescein isothiocyanate (FITC). Suspensions were then incubated together for 1 h before visualization by fluorescence microscopy. Both GBS strains were able to coaggregate with *C. albicans*, indicating that these interactions are not restricted to a single capsular serotype (Fig. 1). Furthermore, as reported previously (12), GBS strain NEM316 exhibited a tropism for *C. albicans* hyphae rather than blastospores. This binding pattern was also apparent with GBS strain 515, although higher levels of association were seen overall with strain NEM316 (Fig. 1). Taken together, these data confirmed that GBS can undergo planktonic interactions with *C. albicans* but implied that levels of coaggregation may be strain dependent.

TABLE1 Microbial strains and plasmids used in this study

Strain or plasmid	Unique ID	Relevant genotype	Reference or source
Strains			
C. albicans			
SC5314	UB1843	Wild type	Neil Gow, University of Aberdeen
	UB1941	Δ*als3*	39
	UB1940	Δ*als3* pUL.*als3*	39
S. cerevisiae			
BY4742	UB2156	pBC542-*ALS3sm*	40
S. agalactiae			
NEM316	UB1931	Wild type	Shaynoor Dramsi, Institut Pasteur
515	UB2410	Wild type	Victor Nizet, University of California, San Diego
515	UB2873	Δ*bspC*	This study
L. lactis			
NZ9800	UB2635	pMSP	12
	UB2658	pMSP.*bspA*	12
	UB2659	pMSP.*bspC*	This study
Plasmids			54
pMSP7517		*E. coli-Enterococcus* shuttle vector containing *Enterococcus faecalis prgB* under the control of the *nisA* promoter; erythromycin resistance	
pMSP.*bspC*		pMSP7517-derived plasmid containing *bspC* from GBS 515 in place of *prgB*	
pR326		Chloramphenicol resistance	58
pHY304		Temp-sensitive *E. coli-Streptococcus* shuttle vector	53

GBS-*C. albicans* interactions with VECs. Since GBS and *C. albicans* are able to coaggregate, we hypothesized that such interactions can influence the capacity of these microbes to associate with vaginal epithelium. To this end, an *in vitro* assay was developed using the vaginal epithelial cell (VEC) line VK2/E6E7. In the first instance, epithelial cell monolayers were exposed either to GBS alone for 1 h or to *C. albicans* for 1 h to initiate hypha formation followed by GBS for a further 1 h. Associated GBS organisms were then enumerated by viable counts (CFU) from epithelial cell lysates. While GBS strain NEM316 showed a higher level of association (1.51 x10^5 CFU/ monolayer) than that of strain 515 (7.57 x10^4 CFU/monolayer) (Fig. 2), both strains exhibited a strong affinity for the VEC monolayers. However, significantly larger numbers of bacteria were recovered for both strains in the presence of *C. albicans*. Numbers of GBS cells recovered from the epithelium were 1.9-fold higher for strain NEM316 and 2.1-fold higher for strain 515 than those from their respective monospecies samples (Fig. 2). These augmented effects were verified by confocal microscopy, although a slightly longer incubation period (5 h) was needed to obtain bacterial cell numbers that were of sufficient abundance to be clearly visible (Fig. 3). For monospecies samples, both GBS strains were evenly distributed across the VECs, but numbers of GBS cells per field of view were higher for strain NEM316 than for strain 515 (Fig. 3, columns 1 and 2). In the presence of *C. albicans*, an increase in the number of GBS cells associated with the VECs was apparent for both strains (Fig. 3, columns 3 and 4) compared to those for the monospecies equivalents. This was verified by quantification of GBS biovolume (Fig. 4A). In the presence of *C. albicans*, GBS biovolume levels were 4.6-fold and 2.8-fold higher for strains NEM316 and 515, respectively, than for their monospecies equivalents (Fig. 4A). Many GBS cells could be seen interacting with *C. albicans* hyphae, which formed extensive mats that overlaid the epithelial monolayers (Fig. 3, white arrows). However, there was also a

visible increase in the number of GBS cells interacting with the epithelium in areas that were not seemingly colonized by *C. albicans* (Fig. 3, red arrows). This pattern was seen for both GBS strains. Augmentation of the GBS association with VECs after 5 h by *C. albicans* was further supported by enumeration of GBS cells from recovered epithelial lysates, and the effects were demonstrated more strikingly with GBS strain 515 (Fig. 4B).

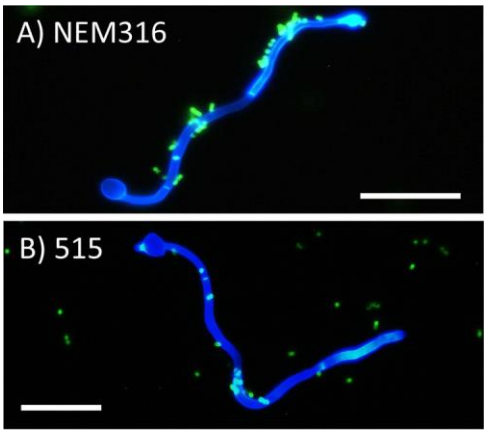

FIG 1 Fluorescence micrographs of planktonic interactions between *C. albicans* and GBS. *C. albicans* SC5314 was grown in YNBPTG for 2 h at 37°C and 220 rpm before addition of GBS strain NEM316 (A) or GBS strain 515 (B) and incubation for a further 1 h. GBS was labeled with FITC (green), and *C. albicans* was labeled with calcofluor white (blue). Bars, 20 µm.

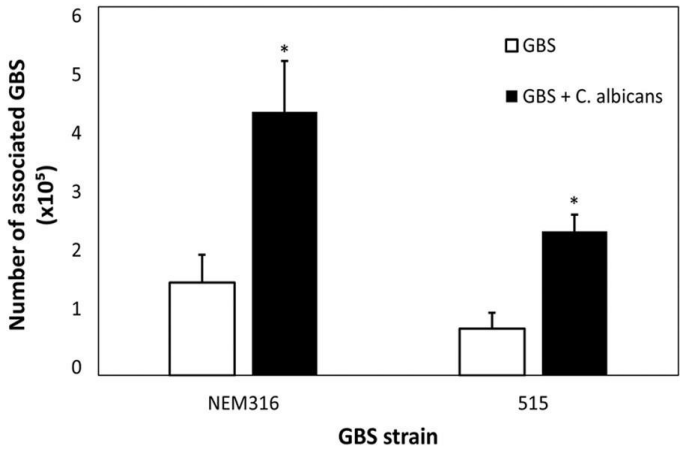

FIG 2 Effects of *C. albicans* SC5314 on association of GBS with VECs. VEC monolayers were incubated with GBS suspensions (MOI 2.5) for 1 h (open bars) or with *C. albicans* (MOI 2.5) for 1 h followed by GBS for a further 1 h (black bars). Monolayers were then lysed, and associated GBS cells were enumerated by serial dilution onto THY agar supplemented with 50 g/ml nystatin. *, P <0.05 compared to monospecies controls, as determined by unpaired Student's t test (n=4).

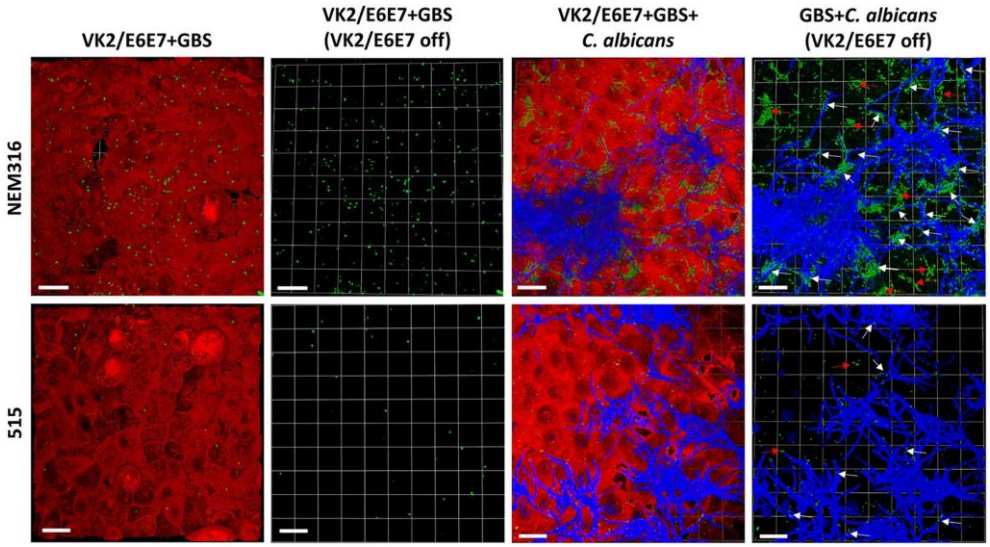

FIG 3 Representative confocal micrographs of *C. albicans*-GBS association with VECs. VEC monolayers were incubated with GBS alone for 5 h (columns 1 and 2) or with *C. albicans* for 1 h followed by GBS for a further 5 h (columns 3 and 4). Cells were then fixed, stained, and mounted onto glass slides. GBS was labeled using an Alexa Fluor 488-conjugated antibody (green), *C. albicans* was labeled with calcofluor white (blue), and VECs were labeled with phalloidin-TRITC (red). GBS strains NEM316 (top panels) and 515 (bottom panels) were tested. Columns 2 and 4 are duplicates of columns 1 and 3, respectively, in which the red filter (i.e., the VECs) has been removed (VK2/E6E7 off). Bars, 100 µm. White arrows indicate areas where GBS binds *C. albicans* hyphae, while red arrows indicate areas where GBS is found in the absence of *C. albicans*.

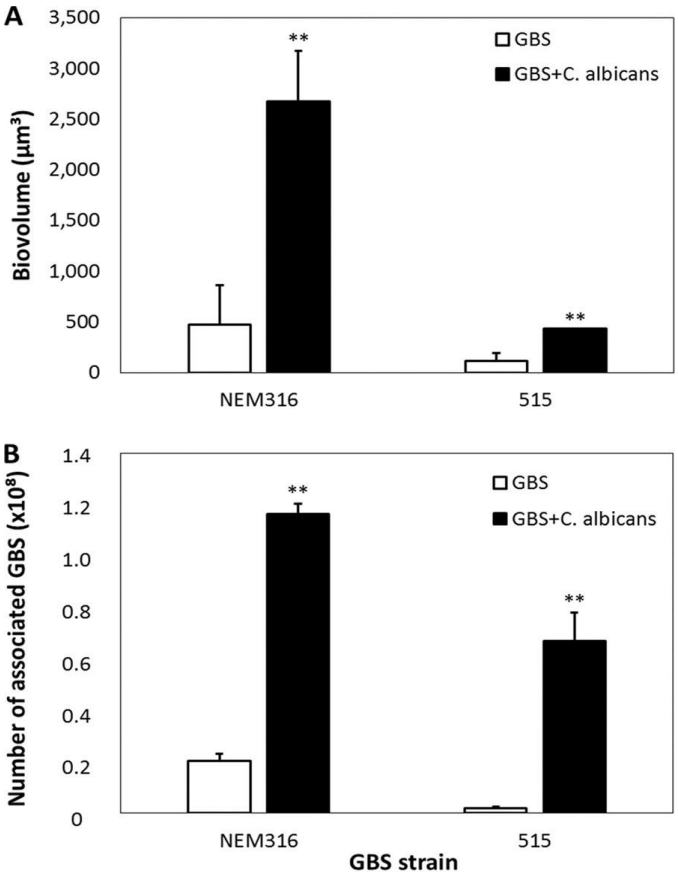

FIG 4 Effects of *C. albicans* on association of GBS with VECs following 5 h of incubation. (A) Quantification of GBS cells from the confocal micrographs illustrated in Fig. 3. Images were processed using Volocity software, and Imaris software was used to calculate GBS biovolumes (in cubic micrometers). (B) Quantification of GBS cells by viable counts. VEC monolayers were incubated with GBS suspensions for 5 h (open bars) or with *C. albicans* for 1 h followed by GBS for a further 5 h (black bars). Monolayers were then lysed, and associated GBS cells were enumerated by serial dilution onto THY agar supplemented with 50 g/ml nystatin. **, $P<0.01$ compared to monospecies controls, as determined by unpaired Student's *t* test ($n=4$).

To investigate the potential for a reciprocal relationship between GBS and *C. albicans*, the effects of GBS on the *C. albicans* association with vaginal epithelium were then explored using the same *in vitro* assay. For both strains tested, the presence of GBS resulted in a 4-fold elevation in the levels of *C. albicans* recovered from the VEC monolayers compared to those of *C. albicans* alone (Fig. 5). These data imply that a synergistic relationship exists between *C. albicans* and GBS and that each microbe can enhance association of the other with the vaginal epithelium.

Role of diffusible signals in GBS-*C. albicans* interactions. One potential mechanism for the enhanced recovery of both GBS and *C. albicans* cocultured with vaginal epithelium might be that each microbe releases some form of diffusible, chemical signal that either stimulates growth of the other or promotes its capacity to associate with VECs. To explore the first possibility, growth levels of GBS and *C. albicans* in singleand dual-species suspensions were compared. These studies were performed under conditions similar to those of the *in vitro* cell culture assay, using keratinocyte serumfree medium (K-SFM) and incubation periods of 1 to 2 h. No significant differences in numbers of CFU were seen for either species (Fig. 6A and B), regardless of whether they were grown under mono- or dual-species conditions. This implies that the presence of *C. albicans* does not affect the overall growth rate of GBS, and vice versa.

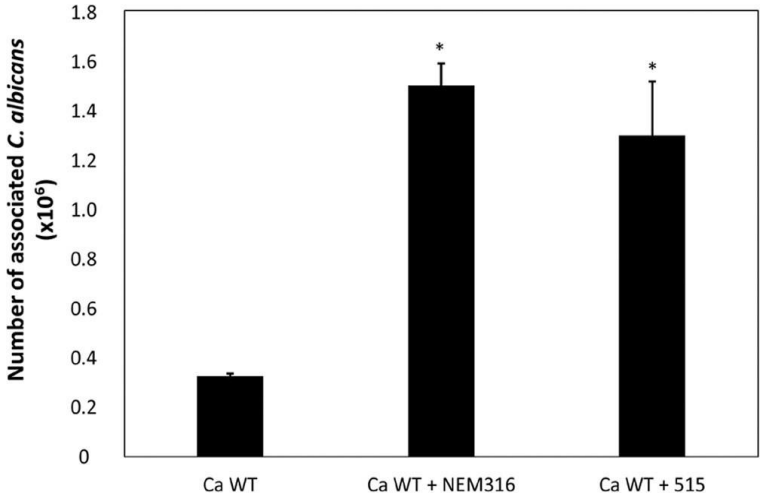

FIG 5 Effects of GBS on association of *C. albicans* SC5314 (Ca WT) with VECs. VEC monolayers were incubated with *C. albicans* cells for 1 h to allow production of hyphae. GBS suspensions were then added for a further 1 h before monolayers were lysed. Associated *C. albicans* cells were enumerated by serial dilution onto SAB agar supplemented with 5 g/ml erythromycin. *, $P<0.05$ compared to the monospecies control, as determined by unpaired Student's *t* test with Bonferroni correction ($n=3$).

To determine if diffusible signals were modulating microbial interactions with the vaginal epithelium, GBS was incubated with VEC monolayers in K-SFM or in spent medium harvested from *C. albicans* grown in K-SFM for 1 h in the presence or absence of VECs. After 1 h of incubation with VEC monolayers, associated GBS cells were enumerated by viable counts from epithelial cell lysates. No significant differences in numbers (CFU per monolayer) of GBS cells recovered were observed across the different conditions (Fig. 7A).

For the reciprocal study, *C. albicans* was incubated on VEC monolayers for 1 h, and then suspensions of GBS or K-SFM alone were placed in transwell inserts above the VECs. Viable counts of *C. albicans* were determined after a further 1 h of incubation. Again, no significant differences in *C. albicans* association levels with VECs were seen in the presence or absence of GBS (Fig. 7B).

One final possibility explored was that GBS or *C. albicans* modulated the permissiveness of VECs to association with the other microbe via an active but contactdependent mechanism. This was investigated by repeating the association assays with paraformaldehyde-fixed VECs. Fixation reduced the numbers of GBS cells recovered from the cell lysates overall. Nonetheless, the presence of *C. albicans* again resulted in elevated association levels of GBS (Fig. 7C), and the reciprocal effect was seen for levels of *C. albicans* recovered in the presence of GBS (Fig. 7D). Taken together, these data imply that neither intermicrobial diffusible signals nor active modulation of the VEC receptor profile is required for enhanced coassociation of GBS or *C. albicans* with the vaginal epithelium.

Role of Bsp protein in GBS-*C. albicans* interactions. Oral streptococci have been shown to promote the colonization and retention of *C. albicans* within the oral cavity, and this is mediated in large part by coadhesion between the microbes (31). Having demonstrated similar coadhesion between GBS and *C. albicans*, the next step was to determine the molecular basis for this physical interaction and its contribution to the synergistic effects seen with vaginal epithelium.

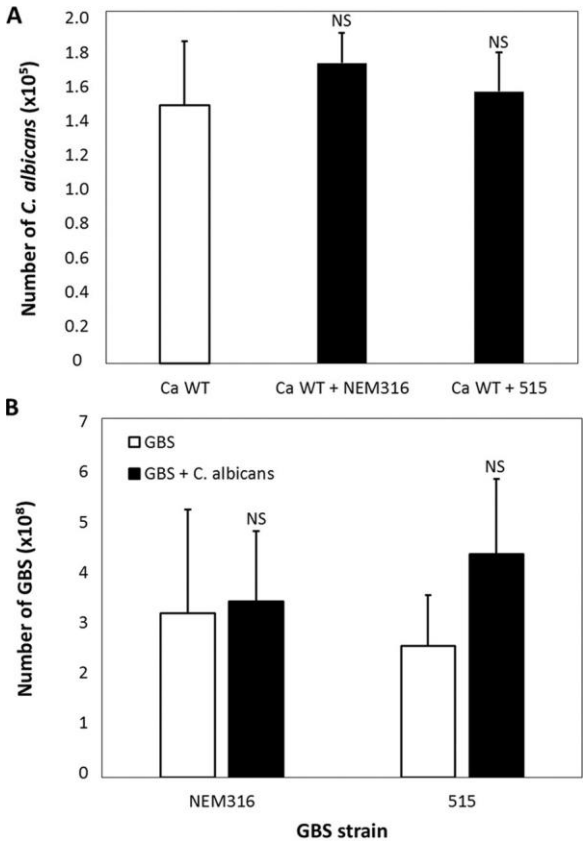

FIG 6 Growth of *C. albicans* or GBS in mono- or dual-species suspension culture. K-SFM broth cultures were inoculated with *C. albicans* SC5314 (Ca WT) at 37°C and 220 rpm for 1 h before addition of GBS and incubation for a further 1 h (black bars). Alternatively, broth cultures were inoculated with *C. albicans* or GBS alone and incubated for 2 h or 1 h, respectively (open bars). The numbers of *C. albicans* CFU per milliliter were then determined by viable counts on SAB agar supplemented with 5 g/ml erythromycin (A), while numbers of GBS CFU per milliliter were determined by viable counts on THY agar supplemented with 50 g/ml nystatin (B). NS, P >0.05 compared to the monospecies control, as determined by unpaired Student's t test (n=3).

We recently showed that the AgI/II family protein BspA of GBS strain NEM316 promotes coaggregation with *C. albicans* under planktonic conditions (12). We therefore wanted to build on this observation and determine if the Bsp adhesin family is important in GBS-augmenting interactions of *C. albicans* with VECs. In the first instance, a Δ*bspC* knockout mutant was generated in GBS strain 515, which carries only a single copy of the *bspC* gene (a homologue of *bspA*). This strain displayed only a modest (ca. 15%) reduction in association with VECs compared to that of parent strain 515 (Fig. 8A). However, it was reported previously that streptococci can compensate for loss of AgI/II family proteins by upregulation of alternative adhesins (38). To further explore the role of Bsp adhesins, inhibition studies were therefore performed using specific antisera. Anti-Bsp sera reduced the association of wild-type GBS strains NEM316 and 515 with VECs by 46% and 63%, respectively, compared to that with preimmune control serum (Fig. 8B). Together these data support previous evidence that Bsp adhesins have the capacity to promote GBS interactions with vaginal epithelium (12), but they indicate that there are additional determinants utilized by GBS for this purpose.

In the presence of *C. albicans*, a more significant difference was seen between the parent strain 515 and the Δ*bspC* knockout strain. *C. albicans* significantly promoted recovery of both GBS strains from the epithelium compared to that with their respective monospecies samples. However, numbers of bacteria recovered were approximately 30% lower for mutant strain 515 Δ*bspC* than for parent strain 515 (Fig. 8A).

These data imply that BspC plays a role in mediating GBS coassociation with *C. albicans*. However, additional adhesins must be involved and may compensate for the lack of BspC in strain 515 Δ*bspC*.

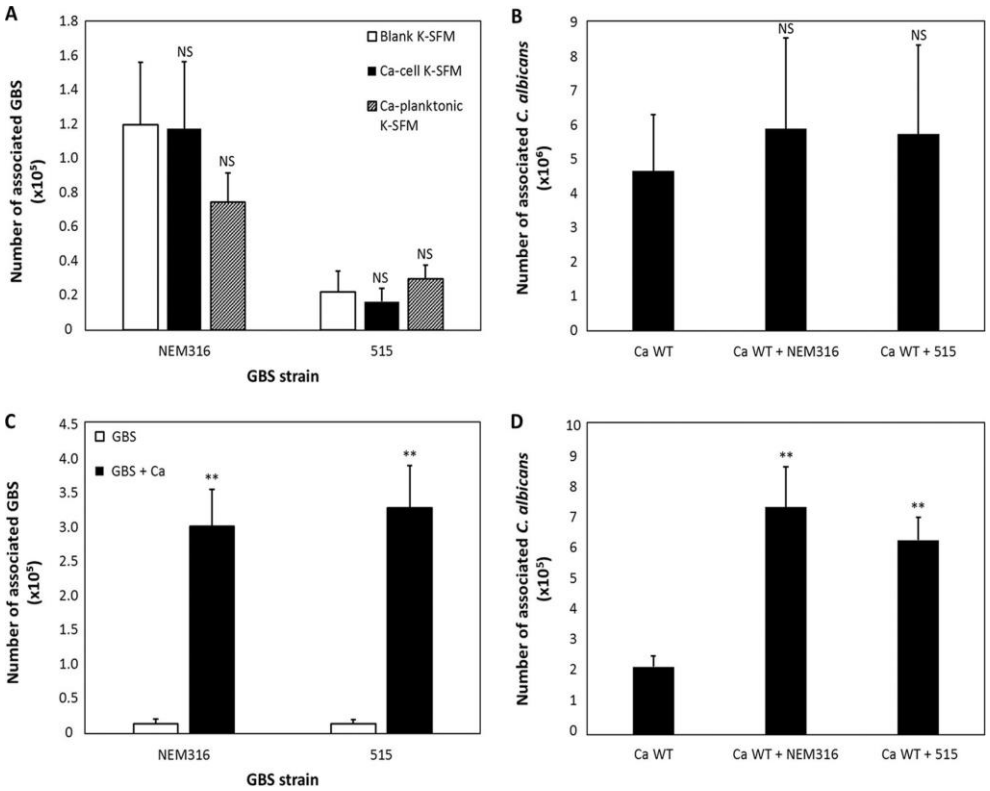

FIG 7 Role of contact-independent mechanisms or fixation in modulating interactions of *C. albicans* or GBS with VECs. (A) *C. albicans* SC5314 (Ca WT) was grown on VEC monolayers, or planktonically in K-SFM medium, for 1 h before spent media were collected and filter sterilized. GBS cells were incubated in these spent media on VECs for 1 h before the monolayers were lysed and associated GBS cells enumerated by serial dilution onto THY agar. NS, $P>0.05$ compared to the blank K-SFM control, as determined by unpaired Student's *t* test ($n=3$). (B) *C. albicans* was grown on VEC monolayers for 1 h before GBS suspensions or K-SFM alone was placed into transwell baskets suspended above. After a further 1 h of incubation, *C. albicans* was enumerated by serial dilution onto SAB agar. (C and D) VEC monolayers were fixed with 2% paraformaldehyde and then incubated with GBS suspensions (MOI 2.5) for 1 h (open bars) or with *C. albicans* (MOI 2.5) for 1 h followed by GBS for a further 1 h (black bars). Monolayers were then lysed, and numbers of GBS CFU per milliliter were determined by viable counts on THY agar supplemented with 50 g/ml nystatin (C), while numbers of *C. albicans* CFU per milliliter were determined by viable counts on SAB agar supplemented with 5 g/ml erythromycin (D). NS, $P>0.05$; **, $P<0.01$ compared to monospecies controls, as determined by unpaired Student's *t* test with Bonferroni correction ($n=4$ [A and B] or 3 [C and D]).

Given this apparent adhesin redundancy, surrogate *Lactococcus lactis* strains expressing BspA or BspC were then employed in coassociation assays. This allowed the functional properties associated with the individual AgI/II family proteins to be explored in greater detail. For monospecies *L. lactis* samples, once again, only a modest increase in numbers of bacteria recovered from VECs was seen for *L. lactis* strains expressing BspA or BspC compared to the numbers with the empty vector control strain (Fig. 9). However, for dual-species samples, recoveries of *L. lactis* strains expressing BspA and BspC were promoted 1.8-fold and 3-fold, respectively, by *C. albicans* (Fig. 9), while vector-only *L. lactis* control recovery was increased only slightly (0.5-fold). Overall this implies that GBS AgI/II family proteins have the capacity to promote

GBS association with vaginal epithelium directly, but they likely play a greater role by promoting association via *C. albicans*.

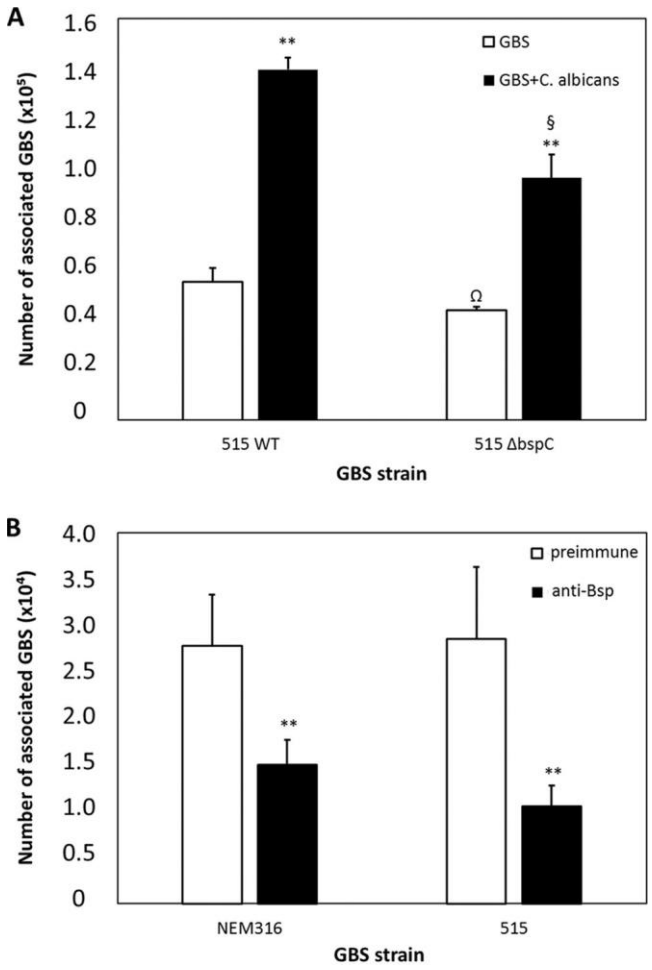

FIG 8 Effects of *C. albicans* or Bsp antisera on the association of GBS wild-type and isogenic mutant strains with VECs. (A) VEC monolayers were incubated with GBS wild-type (WT) strain 515 or mutant Δ*bspC* cell suspensions (MOI 2.5) for 1 h (open bars) or with *C. albicans* SC5314 (MOI 2.5) for 1 h followed by addition of strain 515 suspensions for a further 1 h (black bars). Monolayers were then lysed and associated GBS cells enumerated by serial dilution onto THY agar supplemented with 50 g/ml nystatin. **, significance relative to monospecies controls; Ω, significance relative to wild-type monospecies control; §, significance relative to the wild type in the presence of *C. albicans*. (B) GBS cell suspensions were preincubated with preimmune (open bars) or anti-Bsp (black bars) sera before incubation with VEC monolayers (MOI 2.5) for 1 h and enumeration from cell lysates by viable counts. **, significance relative to preimmune controls. Significance indicates that the *P* value was <0.01 as determined by unpaired Student's *t* test with Bonferroni correction (*n*=3).

Role of Als3 protein in GBS-*C. albicans* interactions. A possible receptor for the Bsp proteins of GBS was the candidal glycoprotein Als3, since this adhesin is hypha specific (22) and has been shown to bind the AgI/II family protein SspB of *S. gordonii* to mediate interkingdom interactions (32). A *C. albicans* strain with both alleles of the *als3* gene deleted (39) and a corresponding complemented strain (Δ*als3als3*) were used to determine if Als3 is involved in interactions between GBS and *C. albicans*. This was first investigated under planktonic conditions, and levels of coaggregation were determined semiquantitatively according to numbers of GBS cells associated with individual hyphae. Both GBS strains exhibited strong interactions with *C. albicans* wild-type strain SC5314 and the *C. albicans* Δ*als3als3* strain, with the majority of hyphae

recorded as binding 6 to 20 bacteria or 20 bacteria (Fig. 10). In contrast, the majority of *C. albicans* Δ*als3* hyphae were either devoid of bacterial cells or bound only 1 to 5 GBS cells (Fig. 10). Thus, the expression of Als3 on candidal hyphae is required to mediate strong physical interactions with GBS under planktonic conditions.

The various *C. albicans* strains were then used to determine if Als3-mediated interactions were required to modulate GBS association with VECs. Interestingly, numbers of *C. albicans* Δ*als3* cells associated with VECs were not significantly different from those recovered for wild-type SC5314 or the *C. albicans* Δ*als3als3* strain. This contrasts with observations made by others (39) in studies of oral epithelium and implies that Als3 may exhibit tissue-specific tropism. Unlike the phenomenon observed with wildtype *C. albicans*, there was no enhanced association of GBS with *C. albicans* Δ*als3* in the presence of VECs, and numbers of GBS cells recovered were comparable to those from monospecies samples (Fig. 11). In contrast, complementation of the Δ*als3* mutation in the *C. albicans* Δ*als3als3* strain restored the capacity of *C. albicans* to significantly promote GBS association with vaginal epithelium relative to that of GBS monospecies samples (Fig. 11).

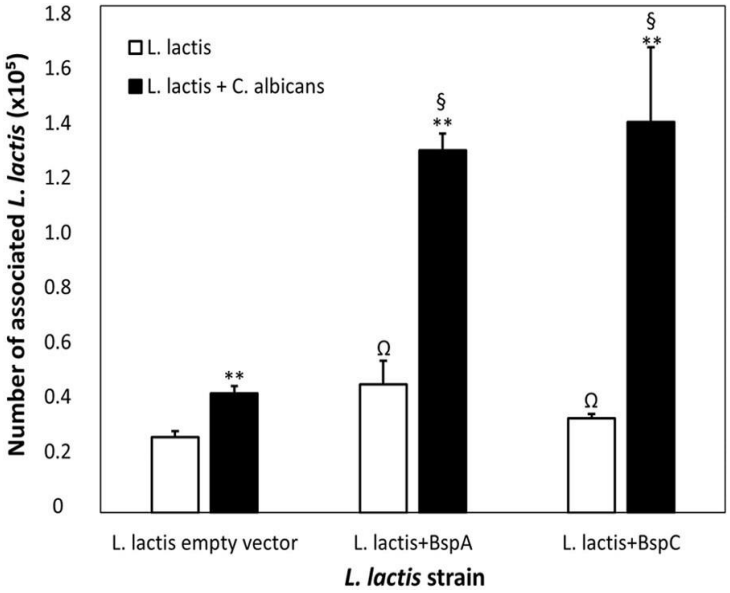

FIG 9 Effects of *C. albicans* on association of *L. lactis* Bsp surrogate expression strains with VECs. VEC monolayers were incubated with suspensions of the *L. lactis* pMSP vector control, pMSP.*bspA*, or pMSP.*bspC* (MOI 2.5) for 1 h (open bars) or with *C. albicans* SC5314 (MOI 2.5) for 1 h followed by addition of *L. lactis* suspensions for a further 1 h (black bars). Monolayers were then lysed, and associated *L. lactis* cells were enumerated by serial dilution onto GM17 agar supplemented with 50 g/ml nystatin. **, significance relative to monospecies controls; Ω, significance relative to the pMSP empty vector control; §, significance relative to the pMSP empty vector control in the presence of *C. albicans*. Significance indicates that the *P* value was <0.01 as determined by unpaired Student's *t* test with Bonferroni correction (*n*=4).

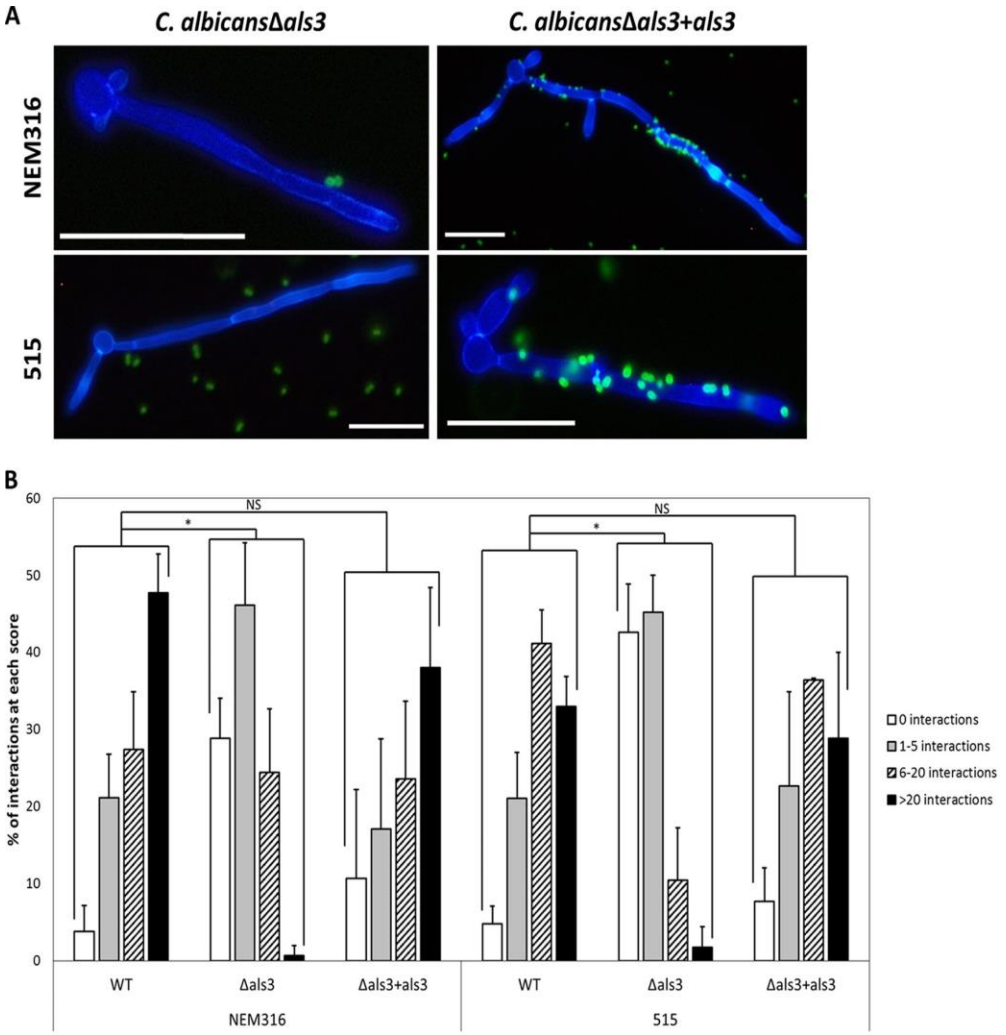

FIG 10 Role of Als3 in planktonic interactions between *C. albicans* and GBS. (A) Fluorescence micrographs of planktonic interactions between the *C. albicans* Δ*als3* strain (left panels) or the Δ*als3als3* complemented strain (right panels) and GBS strain NEM316 (top panels) or 515 (bottom panels). *C. albicans* was grown in YNBPTG for 2 h at 37°C and 220 rpm before addition of GBS and incubation for a further 1 h. GBS was labeled with FITC (green), and *C. albicans* was labeled with calcofluor white (blue). Bars, 20 m. Note that interactions of GBS strains with wild-type *C. albicans* SC5314 are shown in Fig. 1. (B) Semiquantitation of *C. albicans* hyphae with 0, 1 to 5, 6 to 20, or 20 interacting GBS cells, based on approximately 40 randomly selected images for each experimental group. *, $P<0.05$; NS, $P>0.05$ (as determined by linear regression analysis of data sets) ($n=4$).

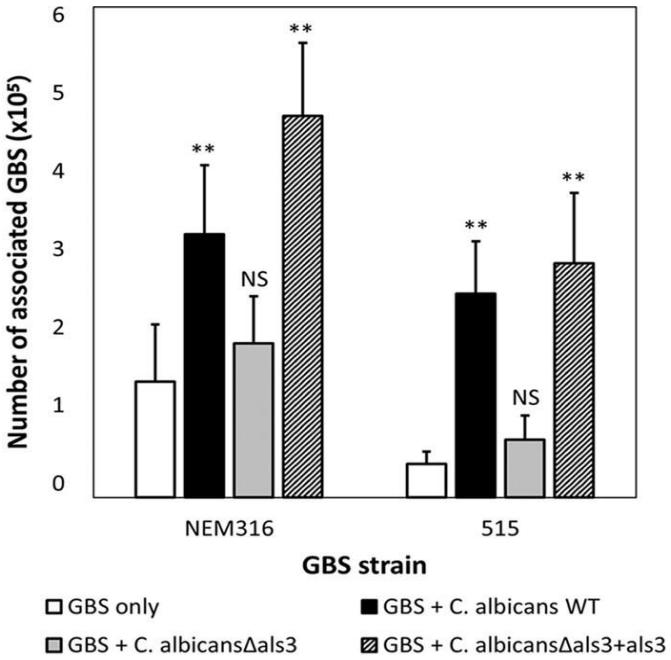

FIG 11 Role of Als3 in synergistic effects of *C. albicans* on association of GBS with VECs. VEC monolayers were incubated with GBS suspensions for 1 h (open bars) or with *C. albicans* SC5314 (wild type [WT]) (black bars), Δ*als3* (gray bars), or Δ*als3als3* (striped bars) for 1 h followed by GBS for a further 1 h. Monolayers were lysed, and then associated GBS cells were enumerated by serial dilution onto THY agar supplemented with 50 g/ml nystatin. **, $P<0.01$; NS, $P>0.05$ (as determined by unpaired Student's *t* test with Bonferroni correction) ($n=4$).

A similar scenario was seen for reciprocal studies to determine the role of Als3 in GBS modulation of *C. albicans* interactions with VECs. Numbers of *C. albicans* Δ*als3* cells recovered from epithelial monolayers were comparable for monospecies samples and dual-species samples incorporating either of the two GBS strains (Fig. 12). In contrast, both GBS strains enhanced the recovery of *C. albicans* Δ*als3als3* 2.5-fold (Fig. 12) relative to that of the monospecies control. These effects were similar to those seen previously with *C. albicans* wild-type strain SC5314 (Fig. 5). Thus, Als3 expression by *C. albicans* is required for both GBS and *C. albicans* to modulate coassociation with vaginal epithelium.

Finally, studies were performed to investigate if Bsp polypeptides of GBS can bind directly to Als3 of *C. albicans*. Again, to avoid potential issues with adhesin redundancy, surrogate expression strains were utilized. A strain of *Saccharomyces cerevisiae* that expresses the small allele of *C. albicans* Als3 on its cell surface was previously generated (40). This *S. cerevisiae* (Als3) strain was fluorescently labeled with FITC, while *L. lactis* strains expressing BspA, BspC, or an empty vector control were labeled with tetramethyl rhodamine isocyanate (TRITC). Suspensions were then incubated together for 1 h before visualization by fluorescence microscopy. No interactions were seen between *S. cerevisiae* (Als3) and the *L. lactis* control (Fig. 13A). In contrast, coaggregation could clearly be seen with *S. cerevisiae* (Als3) and *L. lactis* strains expressing either BspA (Fig. 13B) or BspC (Fig. 13C). Thus, GBS polypeptides BspA and BspC are direct binding partners for Als3 of *C. albicans*.

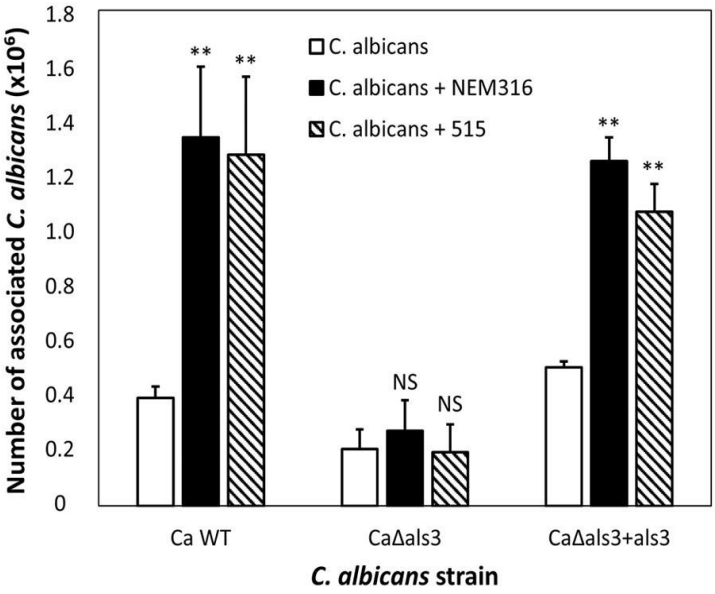

FIG 12 Role of Als3 in synergistic effects of GBS on association of *C. albicans* with VECs. VEC monolayers were incubated with *C. albicans* SC5314 (wild type [WT]) (open bars), Δ*als3* (black bars), or Δ*als3als3* (striped bars) for 1 h followed by GBS for a further 1 h. Monolayers were lysed, and associated *C. albicans* cells were enumerated by serial dilution onto SAB agar supplemented with 5 mg/ml erythromycin. **, $P < 0.01$; NS, $P > 0.05$ (as determined by unpaired Student's *t* test with Bonferroni correction) ($n=4$).

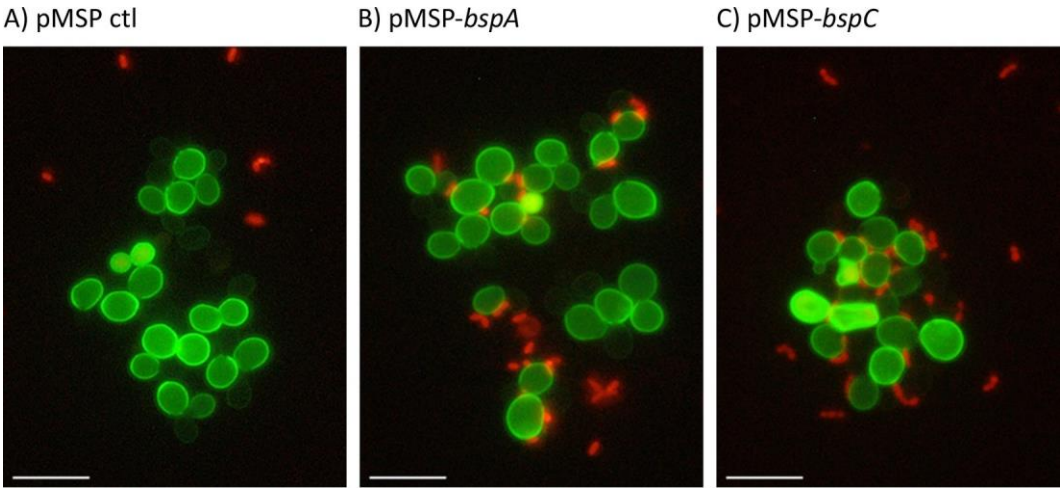

FIG 13 Fluorescence micrographs of planktonic interactions between *S. cerevisiae* Als3 and *L. lactis* Bsp surrogate expression strains. *S. cerevisiae* (Als3) was grown in YNBPTG for 3 h at 30°C and 220 rpm before addition of *L. lactis*(pMSP) (control) (A), *L. lactis*(pMSP.*bspA*) (B), or *L. lactis*(pMSP.*bspC*) (C) and incubation for a further 1 h. *L. lactis* was labeled with TRITC (red), and *S. cerevisiae* was labeled with FITC (green). Bars, 20 µm.

DISCUSSION

Intermicrobial interactions occur at most sites of colonization within the human body, and according to the National Institutes of Health, biofilms underpin approximately 80% of infections (41). In some

instances, these interactions have antagonistic outcomes, such as those between *C. albicans* and *Pseudomonas aeruginosa*. Other
partnerships are seemingly synergistic in nature, such as the interactions between *C. albicans* and *S. gordonii*, *S. oralis*, *S. mutans*, and *S. aureus* (32, 35, 37, 42). Several studies have reported the cooccurrence of GBS and *C. albicans* within the GU tract (14–18), and we recently provided evidence for coaggregation of these two microbes (12). The aim of this study was therefore to further define the interkingdom interactions of these two microbes and their capacity to modulate GU tract colonization, an essential step in the pathogenesis of both microorganisms.

Using the VEC line VK2/E6E7 as a model system, this study provides evidence that a reciprocal, synergistic relationship exists between GBS and *C. albicans* and may serve to promote their cocolonization of the vaginal mucosa. Specifically, when the organisms were incubated together, numbers of both microbes associated with the epithelial monolayers were found to be significantly larger than the numbers recovered from equivalent monospecies samples. Confocal microscopy revealed extensive hyphal "mats" of candidal cells overlaying the epithelial monolayers to which GBS cells were attached. This implies that direct physical contact (i.e., coadhesion) between GBS and
C. albicans is a key mechanism that contributes to their synergistic interplay. Thus, GBS may bind directly to epithelium or to adherent *C. albicans* cells, and vice versa.

To identify the mechanistic basis of coadhesion between GBS and *C. albicans*, our studies focused on the hypha-specific adhesins of *C. albicans*, and specifically the adhesin Als3, since a distinct tropism for candidal hyphae was observed for both GBS strains tested. Use of Als3 knockout and complemented strains of *C. albicans* confirmed that recognition of this glycoprotein by GBS is required for effective coaggregation of these two microbes under planktonic conditions and for coassociation with vaginal epithelium. This correlates well with the interactions of *C. albicans* and streptococci within the oral cavity reported to date (32, 34) and thus may imply that Als3 recognition represents a common mechanism for *C. albicans* engagement by members of the *Streptococcus* genus. The addition of GBS to the list of microbes that utilize Als3 as a receptor, alongside other streptococci, *S. aureus*, and *Rothia dentocariosa* (32, 37, 43), also adds support to the notion that Als3 plays a major role in the capacity of *C. albicans* to mediate a diverse range of polymicrobial interactions.

In addressing the GBS side of this synergistic partnership, this study provides evidence for the role of GBS AgI/II family (Bsp) adhesins in this process. Previous work implicated BspA in facilitating coaggregation of GBS strain NEM316 with *C. albicans* under planktonic conditions (12). These data are supported here and were developed to include the adhesin BspC, implying that these capabilities may represent functions that are shared across the Bsp adhesin family. Moreover, loss of BspC impaired GBS coassociation with *C. albicans*, while expression of BspC by *L. lactis* enabled *C. albicans* to promote association of this surrogate host with VEC monolayers. This extends our current understanding of the properties of the adhesin family and implies that Bsp adhesins are determinants of GBS that facilitate coassociation with *C. albicans* on vaginal epithelium. Moreover, coaggregation of surrogate hosts expressing Als3 and Bsp adhesins adds support to the hypothesis that direct binding between Bsp polypeptides of GBS and Als3 of *C. albicans* is a mechanism that underpins, at least in part, the synergy in epithelial cell interactions between these two microbes. Interestingly, while deletion of *bspC* did not ablate the coassociation of GBS and *C. albicans*, deletion of both *als3* alleles effectively prevented the interaction. This indicates a role for additional GBS determinants in mediating the interkingdom relationship and implies that these determinants may also target the candidal receptor Als3. This supports the evidence that Als3 has the capacity to bind multiple, diverse ligands (44).

Based on primary sequence, the AgI/II family polypeptides of GBS can be divided into four homologues: BspA and -B, which share 90% sequence identity, and BspC and -D, which share 99% sequence identity (12). The highest level of variation between BspA/B and BspC/D is seen within the N-terminal alanine-rich and proline-rich domains. In contrast, the V domain shares 96 to 100% sequence identity across all four Bsp homologues (12). The V domain has been identified as the functional region of a number of AgI/II family polypeptides (45–47), including BspA, in which it was shown to promote binding of GBS NEM316 to the scavenger receptor agglutinin glycoprotein340 (12). If the V domain is also responsible for GBS coassociation with *C. albicans*, then the high level of sequence similarity may explain why both BspA and

BspC display comparable functional properties. Delineating the precise domains within Bsp that are required for engagement with candidal Als3 will be the focus of future studies.

It is clear that direct physical contact between *C. albicans* and GBS plays a significant role in their coassociation with VECs. We also considered the possibility that intermicrobial signals played a role in the processes described here. However, no evidence was found for diffusible molecules released by either *C. albicans* or GBS having the capacity to significantly modulate microbial interactions with vaginal epithelium. Nonetheless, provision of additional intermicrobial binding sites may not be the only mechanism involved in the synergy with VECs. For example, in dual-species images, there were patches of epithelium that were heavily colonized by GBS while seemingly devoid of *C. albicans* (Fig. 3). Fixation of VECs did not inhibit coassociation of GBS and *C. albicans*, implying that these effects are not dependent upon modulation of epithelial cell biology (e.g., receptor availability). Nonetheless, it remains possible that GBS engagement with *C. albicans* alters the GBS receptor profile such that the bacteria are subsequently more permissive to interactions with VECs. The large impact of *als3* gene deletion on the GBS-*C. albicans*-VEC coassociation raises the prospect that Als3 may mediate such effects. Future studies will explore these possible explanations.

To conclude, this study identifies a synergistic interplay between GBS and *C. albicans* that enhances the capacity of both microorganisms to associate with vaginal epithelial cells. Molecular determinants critical to this coassociation mechanism were identified as Bsp adhesins of GBS and Als3 of *C. albicans*. GU tract colonization is an essential first step in the pathogenesis of some diseases, such as vaginal thrush, and is a significant risk factor for neonatal GBS disease due to vertical transmission. Coassociation of GBS and *C. albicans* may therefore have important implications for disease risk for both of these opportunistic pathogens. This coassociation also raises the intriguing possibility of utilizing a convergent immunity approach to develop novel intervention strategies, as has been explored for *C. albicans* and *S. aureus* (48). There is currently no vaccine against GBS disease. Furthermore, while use of intrapartum antibiotic prophylaxis (IAP) has been successful in decreasing the incidence of early-onset neonatal GBS disease in some countries, the logistics of IAP make it an unrealistic control strategy for rural and developing countries, and IAP has had no impact on the rate of late-onset GBS infection (49, 50). The data presented here imply that better control of vaginal colonization by *C. albicans* may restrict or reduce GBS colonization, which in turn would reduce the risk of GBS transmission. Hence, vaccines against *C. albicans*, such as the promising rAls3 vaccine that has completed phase 1 clinical trials (51), may concomitantly help to reduce the burden of neonatal GBS disease.

MATERIALS AND METHODS

Microbial strains and culture conditions. The microbial strains used in this study are listed in Table 1. GBS strains were cultured in Todd-Hewitt broth with 0.5% yeast extract (THY) or on THY agar plates at 37°C and 5% CO_2. *L. lactis* was cultured in GM17 broth (M17 broth supplemented with 0.5% glucose) or on GM17 agar plates at 30°C in a candle jar. *Escherichia coli* was cultured aerobically in Luria-Bertani (LB) broth or on LB agar plates at 37°C. Media were supplemented with 5 g/ml erythromycin or with 50 g/ml (*E. coli*) or 5 g/ml (GBS) chloramphenicol, as appropriate. Heterologous protein expression in *L. lactis* was induced from nisin-inducible plasmids by the addition of 10 ng/ml nisin. Cells from GBS and *L. lactis* broth cultures were harvested by centrifugation at 5,000 *g* for 7 min.

C. albicans was cultured in YPD medium (1% yeast extract, 2% mycological peptone, 2% glucose) at 37°C with shaking (220 rpm) or maintained on Sabouraud dextrose (SAB) agar plates incubated aerobically at 37°C. *C. albicans* cells were harvested from broth cultures by centrifugation at 5,000 *g* for 5 min. *S. cerevisiae* was cultured in complete supplement medium (CSM) without uracil (ForMedium), supplemented with 0.67% yeast nitrogen base (YNB; Difco) and 2% glucose, at 30°C with shaking.

Generation of GBS knockout and *L. lactis* surrogate expression strains. A Δ*bspC* mutant was generated in GBS strain 515 by in-frame allelic replacement with a chloramphenicol resistance gene cassette by homologous recombination, using a previously described method (52). Briefly, a knockout construct was generated by amplifying flanking regions directly upstream and downstream of the *bspC* gene from GBS strain 515 genomic DNA by using primer pairs *bspC*.F1/*bspC*.R1 and *bspC*.F2/*bspC*.R2, respectively (Table 2). A *cat* cassette was amplified from the chloramphenicol resistance plasmid pR326 by use of primers *cat*.F and *cat*.R (Table 2). Upstream and downstream *bspC* and *cat* amplicons were then combined by stitch PCR, using primers *bspC*.F1 and *bspC*.R2. The resultant amplicon was cloned into vector pHY304 (53)

via XbaI and BamHI sites and propagated in *E. coli* Stellar cells (Clontech) prior to isolation and electroporation into GBS 515.

An *L. lactis* strain expressing BspA had been generated previously (12), and a similar methodology was employed here to generate an *L. lactis* strain expressing BspC. In brief, the *bspC* gene was amplified from GBS strain 515 genomic DNA by use of primers pMSP.*bspC*.F and pMSP.*bspC*.R (Table 2). The resultant amplicon was then cloned into the nisin-inducible expression vector pMSP7517 (54) via NcoI and XhoI sites, generating plasmid pMSP.*bspC*. This construct was transformed directly into electrocompetent *L. lactis* NZ9800 as described previously (12). Transformants were confirmed by plasmid isolation and PCR, while expression of BspC in *L. lactis* was verified by dot immunoblotting.

TABLE2 Primers used in this study

Primer name	Sequence[a]
bspC.F1	GCTCTAGAGCAATTAGCAGATGCACAG
bspC.R1	TAAAATCAAAGGAGAAAATATGAACTTTA
bspC.F2	GCTTTTATAATCAATATTCAGAAGCACTTG
bspC.R2	CGGGATCCGAGCCAAATTACCCCTCC
cat.F	AGAAAATATGAACTTTAATAAAATTGATTTAG
cat.R	TGAATATTGATTATAAAAGCCAGTCATTAGG
pMSP.*bspC*.F	CATGCCATGGAGGAGGAAATATGTATAAAAATCAAAAC
pMSP.*bspC*.R	CCGCTCGAGGCAGGTCCAGCTTCAAATC

[a]Restriction endonuclease sites are underlined.

Tissue culture. Experiments were conducted using VK2/E6E7 cells (ATCC CRL-2616), an immortalized human VEC line with a protein profile similar to that of the natural tissue (55, 56). VECs were cultured in K-SFM (Gibco) supplemented with 0.4 mM $CaCl_2$, 0.05 mg/ml bovine pituitary extract, and 0.1 ng/ml epidermal growth factor. Upon reaching 70 to 80% confluence, cells were disassociated by use of TrypLE Express trypsin replacement reagent (Gibco) before being harvested and resuspended in K-SFM. Appropriate volumes of cells were seeded in fresh flasks or in assay plates, as required.

Visualization of dual-species planktonic interactions. Cells from 16-h cultures of *C. albicans* were harvested, washed in YNBPT (1 YNB, 20 mM Na_2HPO_4, 0.02% tryptone, adjusted to pH 7), and suspended to an optical density at 600 nm (OD_{600}) of 1.0 (equivalent to 10^6 cells/ml) in YNBPT. This suspension was diluted 1:10 in YNBPTG (YNBPT supplemented with 0.4% glucose) and incubated at 37°C and 220 rpm for 2 h (2-ml final volume). These growth conditions have previously been shown to induce candidal hypha formation (57).

GBS cells were harvested from 16-h cultures, washed in YNBPT, suspended in 1.5 mM fluorescein isothiocyanate (FITC) dissolved in carbonate buffer (100 mM NaCl, 50 mM Na_2CO_3), and incubated for 30 min with gentle agitation. GBS cells were harvested and washed three times in carbonate buffer, and the pellet was suspended and adjusted to an OD_{600} of 0.5 (equivalent to 5 x10^7 cells/ml) in YNBPTG. GBS suspension (1 ml) was added to that of *C. albicans* and incubated at 37°C for a further 1 h with shaking. Calcofluor white (0.00001% in distilled water [dH_2O]) was added before visualization of 10-l samples by fluorescence microscopy.

For quantification assays, approximately 40 images of randomly selected hyphae were taken for each experimental group. Hyphal interactions were counted and placed into one of the following four groups, similar to a method reported previously (32): 0 interacting bacteria, 1 to 5 bacteria, 6 to 20 bacteria, and 20 bacteria per hypha.

In a variation of this assay, *S. cerevisiae* cells were harvested from a 16-h overnight broth culture in CSM broth, washed once in YNBPT (5 ml), and stained with 1.5 mM FITC for 30 min with gentle agitation. *S. cerevisiae* cells were harvested and washed three times in carbonate buffer. The pellet was suspended and adjusted to an OD_{600} of 1.0 (equivalent to 10^6 cells/ml) in YNBPTG before 1:5 dilution in YNBPTG (final volume, 2 ml). This suspension was incubated at 30°C and 220 rpm for 3 h. *L. lactis* cells were harvested from a 16-h overnight broth culture and washed once in YNBPT before suspension in 2 ml TRITC (0.1 mg/ml in carbonate buffer) and incubation for 30 min with gentle agitation. *L. lactis* cells were harvested, washed three times in carbonate buffer, and adjusted to an OD_{600} of 0.5 in YNBPTG (equivalent to 5 x10^7

cells/ml). The adjusted *L. lactis* suspension (1 ml) was added to *S. cerevisiae* and incubated for a further 1 h at 30°C and 220 rpm before visualization of 10-l samples by fluorescence microscopy.

Microbial growth in dual-species broth cultures. Cells from an overnight (16 h) *C. albicans* suspension culture were harvested and washed once in phosphate-buffered saline (PBS). The pellet was suspended and adjusted to an OD_{600} of 1.0 in K-SFM before being diluted 1:10 in K-SFM (2-ml final volume) and incubated at 37°C and 220 rpm for 2 h. Cells from overnight GBS broth cultures were harvested, washed once in PBS, and suspended in K-SFM to an OD_{600} of 0.5. GBS suspension (1 ml) was added to the *C. albicans* suspension, and the mixture was incubated at 37°C for a further 1 h. Planktonic suspensions were vortex mixed for 15 s before being serially 10-fold diluted in THY broth. Numbers of microorganisms were detected by viable counts (CFU) on either THY agar plates (GBS) supplemented with 50 g/ml nystatin to inhibit *C. albicans* growth or SAB agar plates (*C. albicans*) supplemented with 5 g/ml erythromycin to inhibit GBS growth.

Epithelial association assay. Epithelial association assays were conducted as described previously (5), with a few modifications. VECs were seeded into a 24-well plate at 2 $x10^5$ cells/well and incubated at 37°C and 5% CO_2 until confluent (48 to 72 h). *C. albicans* cells were diluted in K-SFM to obtain approximately 5 $x10^5$ cells/ml, while GBS or *L. lactis* cells were diluted in K-SFM to obtain approximately 5 $x10^5$ cells/ml.

Wells containing VEC monolayers were washed once with PBS, and approximately 5 $x10^5$ bacteria or *C. albicans* cells (1 ml; multiplicity of infection [MOI] 2.5) were then added to each well. Bacterial suspensions were incubated at 37°C and 5% CO_2 for 1 h, while *C. albicans* suspensions were incubated for 2 h. For dual-species assays, *C. albicans* suspensions were incubated for 1 h before the medium was replaced by GBS or *L. lactis* and incubated for a further 1 h. For all assays, wells were then washed three times with PBS before incubation for 15 min with TrypLE, followed by two ice-cold water incubations, lasting 20 min each, to lyse the VECs. Lysates were serially diluted onto THY (GBS), GM17 (*L. lactis*), or SAB (*C. albicans*) agar plates and viable counts determined as described above. It was confirmed both visually and by monitoring levels of lactate dehydrogenase (LDH) released into the culture supernatants that epithelial monolayers remained intact and viable over the periods of the mono- or dual-species association assays.

In a variation of this assay, VEC monolayers were fixed in 2% paraformaldehyde overnight prior to incubation with cell suspensions of *C. albicans* and/or GBS. Alternatively, GBS suspensions were prepared as described above and preincubated at room temperature with 10 g/ml rabbit preimmune or anti-Bsp sera (Eurogentec) for 30 min prior to incubation at 37°C for 1 h with VEC monolayers.

Spent medium studies. VECs were seeded in a 24-well plate and grown to confluence. *C. albicans* was prepared as described above and then incubated with the VECs or grown planktonically in K-SFM medium for 1 h. The *C. albicans* medium was then collected and sterilized by filtration through a 0.2-m filter. GBS suspensions, prepared as described above, were adjusted to an OD_{600} of 1.0 in K-SFM before being diluted 1:200 in either fresh K-SFM, K-SFM from *C. albicans* planktonic growth, or K-SFM from *C. albicans* growth on VK2/E6E7 monolayers. Aliquots (1 ml) were added to VEC monolayers and incubated for 1 h. VECs were disassociated and lysed as described above, and numbers of GBS CFU were determined by serial dilution and viable counts on THY agar plates.

Transwell studies. VECs were seeded in a 24-well plate and grown to confluence. *C. albicans* cells were prepared as described above and then incubated with VEC monolayers for 1 h before the medium was replaced with 1 ml K-SFM. Transwell inserts with high-density, 0.4-m pores (Sarstedt) were placed into wells. GBS suspensions in K-SFM (OD_{600} 1.0) were diluted 1:100 in K-SFM. Aliquots (0.5 ml) were added to the transwell inserts, and the plates were incubated for a further 1 h. The inserts were removed, remaining VECs were disassociated and lysed as described above, and numbers of *C. albicans* CFU were determined by serial dilution and viable counts on SAB agar plates.

Confocal microscopy. For visualization by confocal microscopy, VEC monolayers were grown on 19-mm glass coverslips in a 12-well plate until confluent. The epithelial association assay was then carried out as described above, except that the time was extended by 4 h. Calcofluor white (1 l) was added to stain the chitin in the *C. albicans* cell wall, and the coverslips were then fixed in 2% paraformaldehyde. Triton X-100 (0.3%) was used to permeabilize the epithelial cells before blocking in 2% bovine serum albumin (BSA). Bacteria were stained with a mouse anti-GBS antibody (1.B.501; Santa Cruz Biotechnology) followed by an Alexa Fluor 488-conjugated goat anti-mouse antibody (Fisher), both of which were used at a dilution of 1:200. The F-actin of the epithelial cells was stained with phalloidin-TRITC (Sigma). Coverslips were then mounted onto glass slides by use of Vectashield reagent (Vector Laboratories) and imaged on a Leica SP5-

AOBS confocal laser scanning microscope (CLSM) attached to a Leica DM I6000 inverted epifluorescence microscope. Images were processed using Volocity software, and Imaris v7.5 software (Bitplane AG, Zurich, Switzerland) was used to calculate biovolumes (in cubic micrometers).

Statistical analyses. All assays were performed in triplicate unless otherwise stated. Data were analyzed using unpaired Student's *t* tests with Bonferroni correction, as appropriate.

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